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Research Article

Correlation of PD-L1 and CD133 Expression with Metastasis in Invasive Breast Carcinoma of Luminal B Subtype

Sofian Anwar*[1], Bethy Surjawathy Hernowo[2], Birgitta Maria Dewayani[3], Sri Suryanti[4]

[1234]Departement of Anatomical Pathology, University of Padjadjaran/Hasan Sadikin Hospital Bandung, Indonesia

Abstract:

Background: Breast carcinoma is a cancer with the highest number in women, Luminal B is the highest number of all types of invasive breast carcinoma in the world. Invasive breast carcinoma of luminal B is breast carcinoma with hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive), and either HER2 positive or HER2 negative with high levels of Ki-67. Determining the aggressiveness factor in invasive breast carcinoma is very important. PD-L1 is a checkpoint in the cancer cell immunity cycle that affects the aggressiveness of tumour cells and CD133 is a cancerous stem cell marker that plays a role in proliferation, renewal and invasion of tumour cells. This study aims to determine the relationship of PD-L1 and CD133 expression with metastasis in the invasive breast carcinoma of Luminal B subtype.

Methods: This study was an observational analytic study with a case control design to analyze 40 cases of invasive breast carcinoma Luminal B subtype, then divided into 2 groups, metastasis and nonmetastasis groups of 20 cases respectively. Then, all samples were performed by PD-L1 and CD133 immunohistochemistry staining, and then were associated with metastasis. All data were analyzed statistically, tested with a value of $p < 0.05$ from a significant level then processed with SPSS 24.0 for Windows.

Results: The results of this study indicate that there is a significant relationship between PD-L1 immunoexpression and CD133 immunoexpression with metastasis. High CD133 immunoexpression has the greatest risk for metastasis, compared to high immunoexpression in PD1 (PD-L1 Odds Ratio: OR CD133 = 7.364: 12,667).

C**onclusions:** The more increases in PD-L1 and CD133 immunoexpression showed a greater tendency for metastases, and CD133 had the greatest possible risk of metastatic events

Keywords: Luminal B invasive ductal breast carcinoma, cancer stem cells, PD-L1, and CD133

Introduction

Breast cancer is the most common cancer in women in the world. Globocan had recorded at least 2.1 million of new cases in 2018. Based on data from RSHS information system, invasive breast carcinoma is 55.4% of all breast cancer cases diagnosed and occupying the highest prevalence of all types of invasive breast carcinoma in RSHS during 2017.[1, 2]

Breast carcinoma is caused by several factors including genetic change factors, hormonal and environmental influences, genetic changes in luminal B subtype carcinoma involving several gen, so that routine molecular examination of ER, PR and HER2 is not enough, and additional molecular examination can be used as a determinant of cell aggressiveness.[3]

Metastatic cancer is the leading cause of death in patients diagnosed with breast cancer and other malignant tumours. Lymph node status is used to identify the prognosis, tumour stage, and selection of treatment modalities. Patients without lymph node metastasis have a better prognosis, while patients with positive axillary lymph node status of more than 6 have a higher risk of being able to distant metastasis. The progressivity of metastatic lymphatic pathways involves proliferation of lymphatic vessels (lymphangiogenesis), lymphovascular invasion, and lymph node metastases step by step. [4, 5, 6]

Programmed death ligand 1 (PD-L1, also known as CD274 and B7-H1) PD-L1 functions if there is an interaction with its receptor, programmed death 1 (PD-1, CD279), which is located on the cellsur face as a cell surface protein and expressed in T cells, B cells, and dendritic cells. The PD-1 bond in PD-L1 will trigger signal inhibition, and result in reduced effect or cell differentiation, imbalance between immune cell proliferation, T cell apoptosis, energy and saturation.[7, 8, 9]

Wang, et al, revealed that PD-L1 can activate the growth and metastasis of cervical cancer by activating the ITGB4/SNAI1/SIRT3 pathway signal. Expressions integrated as part of a receptor group on the cell surface strongly correlate with metastasis in several types of cancer, then in the immune function of the checkpoint on PD-L1 as a result of bonding with its receptor, PD-1. A hypothesis that explains PD-L1 may bind directly to integrin receptors to activate intracellular signalling pathways such as the AKT/GSK3β pathway can increase the ability of cells to migrate and be invasive is obtained.[6, 10, 11, 12, 13, 14, 15, 16, 17]CD133 or prominin-1, is a cell with a domain of five surface glycoprotein transmembrane, known as a special biomarker for determining human hematopoietic progenitor cells. CD133 is recognized as a biomarker. It is important to identify and isolate certain cell

subpopulations, namely cancer stem cells found in several types of neoplasms including breast cancer.[18]

Activation of the Hedgehog pathway has been shown to be associated with distant metastases, advanced tumour stages and higher TNM stages. In addition, activation of the Hedgehog pathway can cause EMT stimulation. The PI3K/mTOR pathway integrates various signals and has been shown to interact with the Hedgehog line. Recently, the Hippo pathway, which controls the size of organs during development, has been linked to cancerous stem cells.[9, 19, 20]

Matsubara, et al. Reported the results of his research that inhibiting Hedgehog and mTOR will reduce the growth of CD133 + tumour cells on the L3.6pl cell line.41. Severe hypoxia is often found in breast cancer, so the levels of HIF-1α and HIF-2α proteins with immunohistochemical examination will increase compared to the normal surrounding tissue. Breast cancer needs HIF-1α and HIF-2α for cell growth, and metastasis to lymph nodes and lungs, by inducing EMT breast cancer stem cells to make it easier for migration and invasion.[19-24]

This study aimed to perceive the correlation of PD-L1 and CD133 expression with metastasis in invasive breast carcinoma of Luminal B subtype.

Materials And Methods

Patients' Selection

This study used retrospective analytic observational method with case control study design. All data were analyzed using chi-square test with p value < 0.05 of significant level then processed with SPSS 24.0 for Windows.

Ethical clearance has been approved by Health Research Ethic Commission, Padjadjaran University, with assessment number: 116/UN6.KEP/EC/2019.

Data collection of invasive carcinoma of Luminal B subtype cases by mastectomy from the archive section of the Department of Anatomical Pathology, Faculty of Medicine, Padjadjaran University/Dr. Hasan Sadikin Hospital in the period of January 1, 2015 until December 30, 2018.

The recording of research data is paraffin block numbers, medical record numbers, age, and histopathology types. Data recording of research was also conducted on outpatient medical records and hospitalizations of invasive breast carcinoma patients. Based on the acquisition of these data, they will be grouped based on invasive breast carcinoma of luminal B subtype (HER2 + 3 and HER2 -) metastasis and non metastasis according to the inclusion criteria, then a review of hematoxylin eosin preparations and representative blocks was selected. All of these cases were collected by paraffin blocks, assessed for the integrity of the blocks. Representative paraffin blocks were made by two preparations. The first preparation made from immunohistochemistry PD-L1 and CD133 was then assessed using Olympus CX21 light microscope with 10x and 40x magnification to be performed on immunoexpression.

Immunohistochemistry Assessment and Evaluation

Immunohistochemical (IHC) staining using the labelled streptavidin-biotin complex immunoperoxide, uses One Step Neopoly Detection Kit (Scientific Biogear). The primary antibody used was anti-PD-L1 monoclonal rabbit (28-8 clone, paint No. ab205921, Abcam, Inc., .Cambridge, USA) at a dilution of 1 : 200 and mouse polyclonal CD133 antibody of Elabscience (E-AB- 16223) USA with 1 : 100 dilution as primary antibodies and rabbit anti-PD-L1 monoclonal antibody (clone 28-8, cat No. ab205921, Abcam, Inc., Cambridge, UK). Placentas were used as positive controls externally for PD-L1, respectively. Secondary antibodies used are One Step Neo Poly Detection Kit (Scientific Biogear) IHC staining was performed on 4 μm-thick, FFPE tissue sections manually with antigen retrieval methods (0.01M ethylenediamintetra-acetic acid buffer at pH 6.0) as described previously. Then Mayer's hematoxylin as counterstaining was done.

The assessment of PD-L1 and CD133 using a semi quantitative scoring system (histoscore) expression was carried out by looking at the intensity and distribution of immunohistochemical staining. Tumour cells that show positive PD-L1 and CD133 on cell membranes and/or cytoplasm staining were considered positive. The staining intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The percentage of positively stained cells were scored as 0 (negative), 1 (<20%), 2 (20-50%), 3 (50-80%), or 4 (>80%). Histoscore values are obtained by multiplying the intensity and distribution, then made into two categories. Low histoscore values = 0-4 and High = 6-12. Then, they were assessed using a light microscope Olympus CX21 with magnification 10x and 40x for a proper assessment of the expression by two specialists in anatomical pathology without seeing the clinical description of each sample.

Statistical Analysis

Statistical analysis for categorical data was tested by chi-square test if the Chi-Square requirements were met when fulfilled, then the Fisher Exact test was used for 2x2 table. Chi Square requirements have no expected value of less than 5 by 20% of the table. The significance of the statistical test results is determined based on the value of $p < 0.05$. The significance criteria used are p values, in which $p \leq 0.05$ is significant or statistically significant, and $p > 0.05$ is not significant or not statistically significant. The data obtained were recorded in a special form and then processed with the SPSS version 24.0 for Windows.

Results

Patients' Characteristics

The number of patients in this study who met the inclusion criteria were 40 invasive breast carcinoma of Luminal B subtype samples, consisting of 20 samples of the Metastatic group and 20 other samples included in the Non-Metastatic group.

Table 1 describes the overall subject characteristics of study patients according to age and category, tumour size, grade,

distant metastasis, and stage. The average age of subjects is 49.45 ± 11.288 years with the age category of < 50 years occupying the highest proportion compared to the age group of > 50 years. Patients with the most tumour size are 2-5 cm in size.

The highest number of Grade in the subjects of this study is Grade 3.

Patients with distant metastases, which have a positive category, are only found in 1 subject. The highest number of stadiums is stage 3B.

Table 1. characteristics of research subjects

Variable N = 40	N = 40
Age (years)	
Mean ± Std	Std 49.45 ± 11,288
Median	47.50
Range (min-max)	20.00-73.00
Age Category	
<50 years	21 (52.5%)
> = 50 years	19 (47.5%)
Tumor Size	
<2cm	1 (2.5%)
2-5cm	24 (60.0%)
> 5cm	15 (37.5%)
Grade	
1	1 (2.5%)
2	11 (27.5%)
3	28 (70.0%)
Distant metastasis	
Positive	1 (2.5%)
Negative	39 (97.5%)
Stadium	
2A	8 (20.0%)
2B	9 (22.5%)
3A	3 (7.5%)
3B	19 (47.5%)
4	1 (2.5%)
HER2 status	
Positive (+3)	14 (35%)
Negative (-)	26 (65%)

Noted: Categorical data is presented by number/frequency and percentage while numerical data is presented by mean, median, standard deviation and range.

Table 2 explains the comparisons between characteristics by age and categories, Tumor Size, Grade, Distant Metastasis, and Stadium in the Metastatic and Non-Metastatic groups. In the group of Metastatic patients, the average age was 49.90 ± 10,176 years old with a category of < 50 years old and ≥ 50 years old having the same number of patients. The number of patients with the most tumour size is 2-5 cm. The highest number of Grade in the metastatic group is Grade 3.

Table 2. Comparison between Characteristics of Patients Research on Metastasis and Non Metastasis.

Variable	Group		P value
	Metastasis	Non Metastasis	
	N=20	N=20	
Age (years)			**0.805**
Mean±Std	49.90±10.176	49.00±12.553	
Median	50.00	46.00	
Range(min-max)	30.00-66.00	20.00-73.00	
Age Category			**0.752**
<50 years	10(50.0%)	11(55.0%)	
≥50 years	10(50.0%)	9(45.0%)	
Ukuran Tumor			**0.978**
<2cm	1(50%)	0(0.0%)	
2-5cm	10(50.0%)	14(70.0%)	
>5cm	9(45.0%)	6(30.0%)	
Grade			**1.000**
1	0(0.0%)	1(5.0%)	
2	6(30.0%)	5(25.0%)	
3	14(70.0%)	14(70.0%)	
Distant metastasis			**1.000**
Positive	1(5.0%)	0(0.0%)	
Negative	19(95.0%)	20(100.0%)	
Stadium			**1.000**
2A	4(20.0%)	4(20.0%)	
2B	4(20.0%)	5(25.0%)	
3A	3(15.0%)	0(0.0%)	
3B	8(40.0%)	11(55.0%)	
4			
HER2 Status			**0.008****
Positif (+3)	11(55.0%)	3(15.0%)	
Negatif (-)	9(45.0%)	17(85.0%)	

Noted: numerical data the p value is tested by an unpaired T test. Categorical data on p values are calculated based on the Chi-Square test. The value of significance based on the value of $p < 0.05$. Sign * shows the value of $p < 0.05$, meaning that it is significant or statistically significant.

Patient with distant metastasis was only one patient. The highest number of stages in the metastatic group was stage 3B. The average age in the Non-Metastatic group of patients was 49.00 ± 12,553 years old with the category of < 50 years for the most. The highest number of patients with tumour size was in the 2-5 cm category. The highest number of Grade in the NonMetastasis group was Grade 3. NonMetastasis group did not find any patients with distant metastasis. The highest number of stages in the NonMetastasis group was stage 3B.

PD-L1 and CD133 Immunohistochemical Expression

The immunohistochemical expression of PD-L1 and CD133 was evaluated (shown in Figure 1 and 2, respectively). PD-L1 was highly expressed in 29 of 40 cases (72,5%). There were 31 of 40cases (77,5%) showing high CD133 expression.

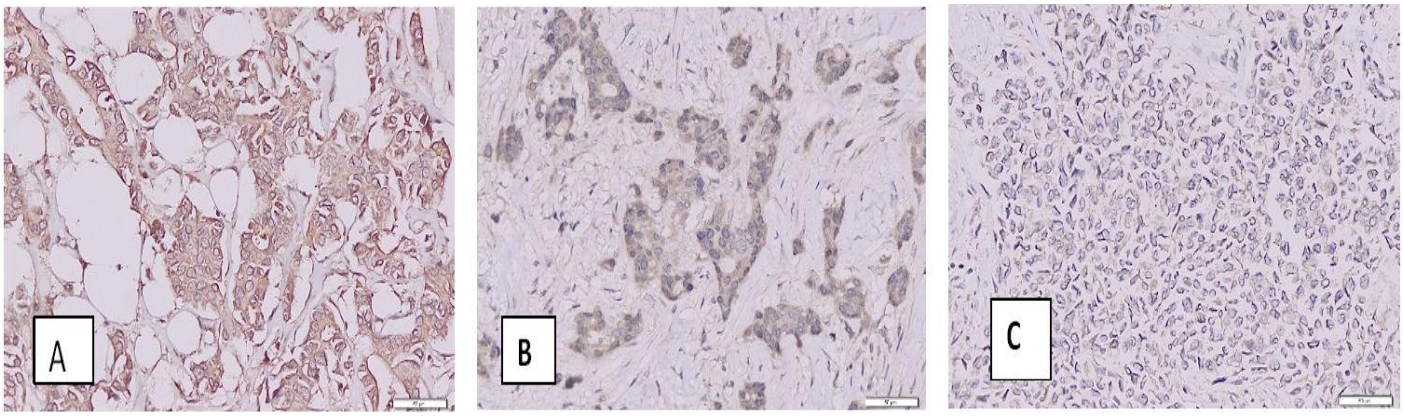

Fig 2. Representative picture of PD-L1 immunohistochemistry. A. Strong (+3) black arrow, B. Moderate (+2) black arrow, C. Weak (+1) black arrow. (200x magnification)

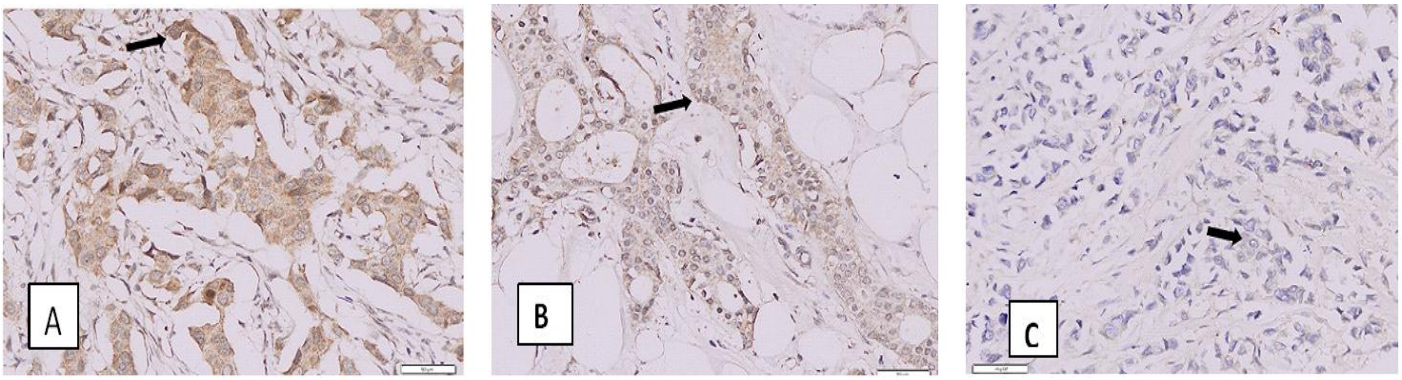

Fig2. Representative picture of CD133 immunohistochemistry. A. Strong (+3) black arrow, B. Moderate (+2) black arrow, C. Weak (+1) black arrow. (200x magnification)

PD-L1 Expression

From the results of statistical tests on the research group in table 3, information was obtained that P value = 0.013 in the PDL-1 Category variable was smaller than 0.05 (P value < 0.05) which meant that it was significant or statistically significant thus it could be explained that there were differences in the proportion of statistically significant between the PDL-1 Category variables in the Metastatic and Non-Metastatic groups. Based on the Odds Ratio, it can be concluded that the possibility of the risk of patients with PDL-1 expression in the high category for metastasis is 7.364 times higher compared to that of the patients with low Category of PDL-1 expression with confidence intervals of 1.337-40.548.

Table 3. Correlation of PD-L1 expression and metastasis in Invasive Breast Carcinoma of Luminal B Subtype

Histoscore	Group NonMetastasis N=20	Metastasis N=20	OR CI (95%)	P value
PD-L1				**0.013****
Low	9(45.0%)	2(10.0%)	**7.364(1.337-40.548)**	
High	11(55.0%)	18(90.0%)		

Noted: The p value is calculated based on the Exact Fisher test. The value of significance based on the p value, $p < 0.05$. Sign * shows the value of $p < 0.05$, meaning that it is significant or statistically significant.

Multivariate Analysis

Multivariate analysis below shows that the two variables between PD-L1 expression and CD133 expression are all related simultaneously to metastasis in invasive breast carcinoma of luminal B subtypes. The risk of the metastatic event will increases if both PD-L1 and CD133 increases simultaneously.

Table 5. Simultaneous correlation of PD-L1 expression and CD133 expression with metastasis in Invasive Breast Carcinoma of Luminal subtype

					OR CI	95% C.I.for EXP(B)	
		B	S.E.	P value	(95%)	Lower	Upper
Step 1[a]	PD-L1	2.195	0.930	0.018	8.978	1.452	55.522
	CD133	2.739	1.171	0.019	15.478	1.558	153.768
	Constant	-5.989	1.903	0.002	0.003		

Discussion

Breast carcinoma is the most common cancer in women in the world, most cases of breast carcinoma are invasive breast carcinoma of Luminal B subtype, and have more aggressive properties and a higher proliferation rate (Ki67> 20%) than Luminal A subtypes.25 One of the important risk factors are age, the higher in age the higher risk, especially after menopause, the risk then increases at 80 years; 75% of women with breast cancer are older than 50 years old, and only 5% are younger than 40 years old. One of the criteria used in relation to prognosis is the aggressiveness of tumour cells characterized by tumour size, histopathological degree, invasion of lymphovascular vessels, tumour cell metastasis and recurrence.25 The results of this study is in accordance with the above theory. Average median age was 47.50 years old (< 50 years old), the highest number of age groups was < 50 years old, the tumour size with the highest numbers in both groups was 2-5 cm and this was also according to the research conducted by Lee, et al. which discovered that primary tumours less than 2 cm in size had a low prognosis for developing metastatic breast carcinoma. Tumour size of 2-5 cm and more than 5 cm were at a very high risk for metastasis.26 Grade with the highest number in both metastatic and non-metastatic groups was grade 3, and the highest number of stages in both groups had the same stages, at stage 3B, this was in accordance with the statement of Zhi-hua Li, et al that patients of breast carcinoma Luminal B had an average age younger than 50 years old, had aggressive properties, tumour size greater than 2 cm and more than 5 cm, and had higher histopathological degrees (2 and 3), and had the ability for lymphovascular invasion.27, 28 Table 1 and 2 of this study provide results in accordance with the theory that is significant in differences between the frequency of HER2+3 with metastatic, P = 0.008, so HER2 is very important role in the pathogenesis in several types of human cancer. HER2 regulates cell growth, survival, and differentiation through signal transduction pathways and plays a role in cell proliferation and differentiation, as expressed by Igbal, N et al. HER2 with homo or heterodimerization produces autophosphorylation of tyrosine residues in the cytoplasmic domain of receptors and initiates various signalling pathways. especially mitogen-activated protein kinas e (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and protein kinase C (PKC) resulting in cell proliferation, survival, differentiation, angiogenesis, and invasion.29,30 Lindsey, et al suggested that HER2 amplification also correlated significantly with the degree of disease, axillary lymph node involvement, histology type, absence of estrogen and progesterone receptors. 30 Immunoediting is a maladaptive reciprocal process. Immune checkpoint of T cells involved in autoimmunity absorbers in the peripheral effector phase of T cell activation is PD-1 (CD279), which leads to immune tolerance of tumour cells expressing PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC). Programmed death ligand 1 (PD-L1) is a 40-kDa transmembrane protein encoded by the CD274 gene located on chromosome 9. PD-L1 is found in several types of malignancies including breast carcinoma.28, 31 The metastatic group in this study showed an increase in the histological score of PD-L1 expression and showed a significant relation with metastasis statistically. The higher the expression of PD-L1, the higher the cell's ability to migrate and metastasis, so that the results are consistent with the theory of the role of PD-L1 on metastasis. The results of this study correspond to the research of Karnik, et al which has concluded that the relationship between the expression of PD-L1 with a parameter of clinicopathologic and aggressiveness including tumour size, the degree of histopathology, invasion of vessels, estrogen receptor, progesterone receptor, HER2, Ki67, types of molecular and triple negative status.32 Wang, s, et al. said that there was a significant relationship between PD-L1 expression and tumour cell metastasis to lymph node.6 While Wang, Q et al., show the results of their research that PD-L1 expression is a poor prognostic factor in several solid tumours and significantly associated with prognosis and survival of patients.33 While Chen, Y. et al disclose the results of their research that PD-L1 can serve as a potential marker for the invasion and prognosis.34 The increasing regulation of PD-L1 selectively very quickly controlled by HIF-1α, is characterized by the expression of PD-L1 in macrophages, dentritic cells, and tumour cells which significantly increased, thereby facilitating tumour cells for migration and invasion. Another theory that supports the role of PD-L1 in the process of metastasis is that the crosstalk between TGFβ and the HER2/Race /MAPK signal which causes secretion of growth factors and cytokines, including TGFβ, which will stimulate EMT and tumor cell invasion. Activation of the AKT / mTOR pathway oncogen will induce PD-L1 expression and then stimulate tumour cells to avoid the immune system. 7, 16, 17 In addition to its ability as pluripotent cells, breast cancer stem cells have been shown to be involved in a fundamental process of tumour development, cell proliferation, and metastatic spread. Besides that, breast cancer stem cells have the capacity for self-renewal, which makes the SPKP immortal, resulting in

recurrence and resistance to various chemotherapies. CD133 is one of the breast cancer stem cells markers.19, 20 CD133 plays an important role as a marker of breast cancer stem cells because it correlates with the size of the tumour, the ability of metastasis, and the clinical stage, so that it can be used as a marker of aggressive tumour cells, and is very useful in establishing diagnosis and can also be used as an indicator of recurrence of malignancy.19, 20 The metastatic group in this study showed a higher increase in the frequency of CD133 expression than that of the Non-Metastatic group, and there was a statistically significant relationship between the expression of CD133 and metastasis. This is consistent with the theory of the role of CD133 as having a role in the process of metastasis. The results of this study are in accordance with what has been done by Liou, et al. that CD133 is associated with cell proliferation as a marker of the aggressiveness of tumour cells.35 Kim, S. J, et al. also proposed that there was a significant relationship between the expression of CD133 and metastases to lymph nodes, chemotherapy and poor prognosis.22 The results of this study are also similar to those proposed by Liou, G.-Y, et al., that cancer cells with CD133 positive are not only capable of self-renewal, proliferation, but also very capable of metastasis and resistant to therapy.42 Tume, L. et al prove their research that a specific subpopulation of CD133+ has been identified in tumour metastasis and CD133 expression in pancreatic carcinoma related to histologic type, lymphatic invasion, lymph node metastasis.36 Schmohl, Jörg U, et al. said that the incidence of malignancy in solid or hematopoietic tumors and recurrence after perfect remission was caused by the presence of a small but very influential population of cancer stem cells, so that appropriate treatment was needed, one of which was to make CD133 as therapeutic target stem cells but were still in the early stages of the clinical phase.23 Based on the results of multivariate analysis, there was a simultaneous significant relationship between PD-L1 expression and CD133 expression with the incidence of metastasis in Invasive Carcinoma of Luminal B subtype. Based on p-value, the little bit smallest one is PD-L1 expression, so that PD-L1 expression has the effects of incidence of metastasis little bit higher than CD133 expression, but based on odds ratio values, CD133 has the highest odds ratio value of 15.478, which means that CD133 expression has a higher risk of 15.478 times for metastasis in Luminal B invasive breast carcinoma (PD-L1 : CD133 Odds Ratio) = 8.978 : 15.478), so that high expression in CD133 has a higher risk for metastasis compared to that of PD-L1. Results of multivariate analysis is consistent with the results of the research of Raniszewska, et al. that the expression of PD-L1 positive stem cells CD133 positive tumors was observed in patients with metastatic p significant results. Breast cancer stem cells with positive PD-L1 expression on metastatic lymph nodes show the immunogenic potential of cancer stem cells, thereby increasing the aggressiveness of cancer stem cells because they can escape from cytotoxic T cells in the immune system.37 Wu, Y, et al. revealed the results of their study that complied with the results of our study that there was an evidence that PD-L1 expression increased in cancerous stem cells in at least two cancer cells, and could be a promising factor to consider immunotherapy based on PD1/PD-L1 to achieve effective and efficient treatment results.38 The increasing number of Luminal B subtypes of invasive ductal carcinoma cases with a higher aggressiveness factor than invasive ductal breast carcinoma Luminal A subtype causes a high incidence of recurrence and resistance to chemotherapy, so that the morbidity and mortality will increase. An evidence based on the role of PD-L1 as an immune checkpoint and CD133 as a marker of stem cells of breast cancer to the process of metastasis of tumour cells of breast, has been shown to contribute significantly to the factor of aggressiveness of the tumour cells, so that PD-L1 is needed as one checkpoint of immunity marker, and also CD133 as breast cancer stem cell marker, so that it can be used as a target therapy and as a prognosis marker too.18, 35 In summary, there was a statistically significant relationship between the high expression of PD-L1 and CD133 to the incidence of metastasis and the possible risk of metastasis in patients with Invasive Breast Carcinoma of Luminal B subtype greater in high CD133 expression states (OR: 12,667) than high PD-L1 expression (OR: 7,364) and the risk becomes greater if it occurs simultaneously (PD-L1 : CD133 Odds Ratio) = 8.978 : 15.478).

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Conflict of Interest

The authors declare that there are no conflict of interests in this study.

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