Vimentin Expression in Stem Cell-like Subtypes of Triple Negative Breast Cancer (TNBC)

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Abstract: Triple negative breast cancers (TNBC) tested phenotypically negative for estrogen (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER-2). This cross-sectional study was performed on breast cancer patients in Haji Adam Malik Hospital Medan from 2013 to 2016. Data about demographics were extracted from patients' records and histopathologic features were obtained using Hematoxylin Eosin (HE) and immunohistochemistry staining. By using CD44, CD24, Twist, Claudin7, and Vimentin, a total 67 breast tumor samples with TNBC were classified as 19 cases of stem-cell like and 48 non stem-cells like subtypes. Postmenopause women with tumor size more than 5cm, higher stage and grade histology were likely to have non stem-cells like subtypes. Women with mucinous carcinoma and metaplastic carcinoma of no special type tended to have non stem-cells like subtypes. Both stem-cells like and non stem-cells like subtypes commonly had low Vimentin. By using immunohistochemistry staining, TNBC can be differentiated into stem-cell like and non-stem-cell like subtypes. Stemness in stem cell like subtypes are resistant to therapy. Therefore, identification of stem-cells in TNBC needs special attention assist in more optimal handling.

1 INTRODUCTION

Breast cancer is the second commonly found cancer and one of the leading causes of death from cancer worldwide for females.[1] Based on data from Oncology Division in Haji Adam Malik Hospital in Medan from 2011 to 2015, an estimated 600 new cases of breast cancer were found each year in Department of Oncology. Breast cancer has heterogenous histopathological features and molecular expressions. Breast cancers tested phenotypically negative for ER, PR, and HER-2 will be called as TNBC.[2] TNBC tends to be more aggressive and until now there has been a disagreement about the treatment because TNBC is not effective to hormonal and targeted anti-HER2 therapy.[3] This subtype has variety of clinical manifestations, histopathological features and molecular expressions, some of which are high grade, with high proliferation rates, grow aggressively and have poor prognosis.[4]

By gene expression profiling, TNBC is classified into 2 major parts, such as basal-like and claudin low.[5] Basal-like subtype is expressed with ER^-, $HER2^-$, $EGFR^+$ and/or Ck5/6; meanwhile $Claudin^{low}$ is expressed with lacking of luminal epithelial differentiation markers (claudin-3, claudin-4, claudin-7, and E-cadherin) and increasing of epithelial-mesenchymal transition markers (EMT) and cancer stem cell (CSC) characteristics ($CD44^+/CD24^{-/low}$).[5,6] This supports that $claudin^{low}$ is cell deriving from immature progenitor cells or stem cells.[7] Although $Claudin^{low}$ and basal-like subtype looks alike, but these two subtypes is completely different.[8] EMT naturally occurs during early embryogenesis phases. But this process also occurs during formation, growth and tumour metastases to distant area. In breast epithelial cells, EMT is related to the invasion of breast cancer cell and mesenchymal character, which is marked with high expression of vimentin.[9,10]

Breast cancer stem cells/BCSCs play a significant role in the growth and development of breast cancer, resistance to therapy, and metastasis.[11] To isolate and identify CSC from other tumous, scientists can use various stem cell markers, such as CD44 and CD24. CD24 is a little more expressed in progenitor cells than in differentiated cells.[12] Therefore, for therapy to be effective, CSC must be recognized and must be differentiated from normal

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breast stem cells. Hence, we were interested to identify vimentin expression in stem-cell-like subtypes of TNBC.

2 METHODS

This descriptive cross-sectional study was carried out in Department of Anatomical Pathology in Haji Adam Malik Hospital and Medical Faculty USU Medan and also in Department of Oncology/Surgical Haji Adam Malik Hospital Medan from March to October 2017. The research was done after permission from Ethical Committee of Medical Faculty USU Medan is granted. Studied population are patients histopathologically diagnosed with breast cancer. Clinical data such as age, tumour size and clinical stage were obtained from medical records and histopathological review of slide were done based on Bloom and Richardson methods modified by Elston Ellis (subtypes and grading histology). ER (clone 6F11, dilution 1: 100, Dako), PR (clone PgR 636, polyclonal Ab, dilution 1: 200, Dako), and Her-2 immunohistochemically staining (clone A0435, polyclonal Ab, dilution 1: 200, Dako) were done. ER and PR were evaluated according to ASCO/CAP guidelines. Tumours were scored as positive for ER and PR (≥1% of nuclear tumour cells stained).[13] Her-2 was considered positive if strongly and homogenous membranous staining/chicken wire pattern (score 3+). If weakly or negative (score 0 or 1+), and score of 2 if membrane cells were incompletely homogenous stained (borderline/moderate). Tumours were defined as TNBC if ER (-), PR (-), and HER2 (-). TNBC tumours were further stained with CD44 (DF1485, dilution 1:100, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), CD24 (C-20, dilution 1:100, Santa Cruz Biotechnology, USA), TWIST-1 (H-81, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz CA), Claudin-7 (NBPI-35677, Rabbit polyclonal antibody, dilution 1:100, Novus Biological), and Vimentin (VIM 3B4, Mouse monoclonal, 1:400, Dako).

Interpreting immunohistochemical stains of CD44 and CD24 were based on Ricardo et al. (2011). CD44, CD24, Twist-1, and Claudin-7 were stained in membrane cells, but Vimentin in cytoplasm. CD44, CD24 and Twist-1 were scored as 0 if no staining or only positive in <10% tumour cells; 1 if 10-25% tumour cells; 2 if 25-50% tumour cells; and 3 if >50% tumour cells.[14] Claudin-7 staining was scored as 0 if no membranous staining; 1+ (1-10% tumour cells); 2+ (10-30% tumour cells); and 3+ (>30% tumour cells).[15] Meanwhile based on percentage, Vimentin were scored as 0 if negative staining; 1 if <30% tumour cells; 2 if 30-60% tumour cells; and 3 if >60% tumour cells. All intensity of staining was scored as 0 if unstained, 1 if weakly stained, 2 if intermediate, and 3 if strong. Interpretation of CD44, CD24, Twist-1, Claudin-7 and Vimentin staining were determined based on multiplication of the percentage of positive cells and the intensity of staining. CD44 and CD24 were scored as 0 negative if (-), 1-3 (+1), 4-6 (+2), and 7-9 (+3).[14] While Twist-1, Claudin-7 and Vimentin was considered weak if total score <6 and strong if total score >6.[16] TNBC was classified as Claudin^{low} (stem cell-like) when CD44^{+}, but if CD44^{-}, then TNBC was classified as non- stem cell-like subtypes. Then, stem cell-like and non-stem-cell like subtypes of TNBC were assessed based on Vimentin expression.

3 RESULTS

In order to determine ontogeny and differentiation of TNBC subtypes in stem cells stages, we used CD44 immunohistochemistry stains. In this study, researchers try to schematically illustrate ontogeny and differentiation of breast epithelial from stem cells to luminal cells with various TNBC molecular markers (Figure 1).

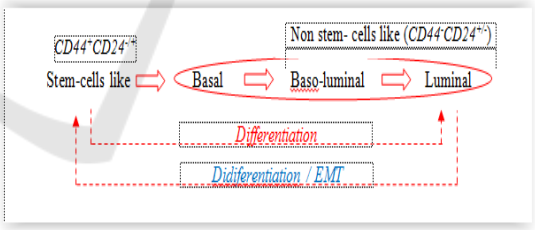

Figure 1 : Ontogeny of stem-cells like, basal, baso-luminal, and luminal subtypes.

From 67 TNBCs in this study, through CD44, CD24, Twist-1 and Claudin-7 immunohistochemistry stains, TNBC was classified as 19 cases of stem-cell like and 48 non stem-cells like subtypes (Table 1). Postmenopause women with tumor size more than 5cm, higher stage and grade histology were likely to have non stem-cells like subtypes of TNBC (86.7%, 71.6%, 71.7%, and 76%, respectively). Women with mucinous carcinoma and metaplastic carcinoma of no special type tended to

have non stem-cells like subtypes of TNBC (100% and 100% respectively) (Table 2). Both stem-cells like and non stem-cells like subtypes of TNBC commonly had low Vimentin (84.2% and 87.5%, respectively). Only 3 cases (12.5%) of stem-cells like and 6 cases (14%) of non stem-cells like subtypes were found with high Vimentin (Table 3).

4 DISCUSSION

The objective of this study is to identify stem cells in breast cancer through immunohistochemistry stains. CSCs or cancer initiating cells (CICs) is a minority in cell populations derived from transformation of self-renewing stem cells, which initiations and maintains growth of cancer cells. There are various of 'stemness' used in identifying BCSCs, for a couple CD44 and CD24.[17,18] In this study we tried to schematically illustrate the ontogeny of stem cells using CD44 and CD24.[19] BCSCs expresses high CD44 and negative/low CD24 ($CD44^{+}CD24^{-/low}$). $CD44^{+}CD24^{-/low}$ fenotype is often related to poor prognosis.[20] CD44 is strong to expressed in immature stem cell and will get weaker on differentiation, whereas CD24 is strongly expressed in more mature cells. The present study demonstrated that the prevalence stem cell like subtypes was 28,3% of all tumours. This result was in opposite to Makki, et al. (2015). They found that the prevalence is 73.7% of all tumours.[21]

Twist displays 'stemness' and plays a role in EMT transformation. Twist is a E-cadherin repressor protein which stimulates EMT. EMT is characterized by lack of keratin epithelial and E-cadherin expression, but expressed Vimentin. During EMT, epithelial cells lose cell-cell contacts, and obtain a mesenchymal morphology.[22] On the other hand, Claudin, an adhesion molecule found in untransformed epithelial cells, is strongly positive in mature differentiated epithelial cells. In this study, CD44, CD24, Claudin-7 and Twist-1 were used as molecular markers of TNBC stem cell-like subtypes. Results from 67 TNBC showed marked heterogenous and overlapping profiles. To demonstrate EMT phenomenon, we also applied vimentin in this study.

Identification of BCSCs gets special attention to date because of having implications for its treatment. Standard chemotherapy often fails because BCSCs have low proliferation and are resistant to chemotherapy which also cause the enhancement of stem cells count. This is one important cause of therapy failure and recurrence in TNBC. Therefore, validation of stem cells in TNBC is mandatory. This is one of the critical steps to develop an effective targeted therapy in TNBC.

Several studies stated that CD44 expression was related to higher histological grade, tumour growth, lymph node invasion and visceral metastases.[23] This results is in accordance with our study. Mesenchymal-like CSCs with $CD24^{-}$ $CD44^{+}$ are primarily quiescent and this condition tends to have high invasive capacity. EMT may be regulated by the tumor microenvironment, such as TGFβ and IL-6.[22] But in this study, researchers found that only 3 cases (12.5%) of stem-cells like and 6 cases (14%) of non stem-cells like subtypes were found with high vimentin.

Table 1 : Classification of stem-cells like subtypes based on immunohistochemistry staining.

No	CD44	CD24	Claudin 7	Twist	Vimentin	Classification of stem cell like subtype
1	+1	+2	0	3	0	SC
2	+1	0	0	0	2	SC
3	1+	+3	7	0	1	SC
4	1+	+3	8	2	1	SC
5	1+	0	7	2	2	SC
6	1+	0	4	3	4	SC
7	1+	3+	8	5	9	SC
8	1+	3+	8	3	3	SC
9	1+	0	8	2	2	SC
10	1+	0	8	2	6	SC
11	1+	3+	8	2	0	SC
12	1+	0	8	0	0	SC
13	1+	0	8	0	1	SC
14	1+	3+	8	2	2	SC

15	1+	3+	5	0	4	SC
16	3+	0	8	0	6	SC
17	2+	0	8	4	0	SC
18	1+	0	7	4	1	SC
19	1+	3+	8	4	2	SC
20	0	+3	8	4	2	NSC
21	0	+3	8	4	2	NSC
22	0	+1	0	3	2	NSC
23	0	+3	8	0	1	NSC
24	0	+3	8	3	0	NSC
25	0	+3	8	2	1	NSC
26	0	+3	7	0	0	NSC
27	0	0	7	0	1	NSC
28	0	+3	8	4	2	NSC
29	0	+3	6	0	1	NSC
30	0	0	7	0	1	NSC
31	0	0	8	0	2	NSC
32	0	+3	8	0	3	NSC
33	0	+3	0	0	0	NSC
34	0	+3	8	2	0	NSC
35	0	+3	8	0	2	NSC
36	0	+3	8	0	1	NSC
37	0	+3	4	0	3	NSC
38	0	+3	**8**	0	3	NSC
39	0	+3	0	0	0	NSC
40	0	2+	0	0	0	NSC
41	0	3+	8	**2**	2	NSC
42	0	3+	7	0	2	NSC
43	0	3+	0	0	1	NSC
44	0	3+	8	0	2	NSC
45	0	3+	7	2	2	NSC
46	0	3+	8	2	1	NSC
47	0	3+	7	3	2	NSC
48	0	3+	8	0	1	NSC
49	0	3+	8	2	2	NSC
50	0	3+	7	0	2	NSC
51	0	0	8	3	0	NSC
52	0	3+	8	4	3	NSC
53	0	0	8	2	9	NSC
54	0	2+	8	0	2	NSC
55	0	0	8	3	2	NSC
56	0	2+	8	0	6	NSC
57	0	1+	8	3	6	NSC
58	0	0	8	3	2	NSC
59	0	3+	8	0	3	NSC
60	0	0	7	2	9	NSC
61	0	0	8	0	0	NSC
62	0	0	6	6	6	NSC
63	0	3+	8	0	1	NSC
64	0	0	7	0	2	NSC
65	0	0	5	4	0	NSC
66	0	3+	7	5	9	NSC
67	0	2+	8	0	2	NSC

Table 2 : Classification of stem-cells like subtypes based on clinicopathological characteristics.

Variables	Classification of stem cell like				Total
	Stem cell like	%	Non stem cell like	%	
Status menopausal					
• Premenopause	17	32.7	35	67.3	52
• postmenopause	2	13.3	13	86.7	15
Tumor size					
• 2-5cm	1	33.3	2	66.7	3
• >5cm	18	28.1	46	71.6	64
Stage					
• II	6	28.6	15	71.4	21
• III	13	28.3	33	71.7	46
Grade					
• II	13	31	29	69	42
• III	6	24	19	76	25
Subtype histology					
• IC-NST	17	30.9	38	69.1	55
• ILC	1	25	3	75	4
• Mucinous carcinoma	0	0	1	100	1
• Carcinoma with medullary features	1	20	4	80	5
• Metaplastic carcinoma of no special type	0	0	2	100	2
Total	19	28.4	48	71.6	67

Table 3 : Classification of stem-cells like subtypes based on Vimentin expression.

Classification of stem cell like	Vimentin				Total
	low	%	high	%	
Stem cell like	16	84.2	3	15.8	19
Non stem cell like	42	87.5	6	12.5	48
Total	58	86.6	9	13.4	67

5 CONCLUSION

TNBC is a heterogenous breast cancer. By using immunohistochemical staining panels, TNBC can be classified as stem cell like and non-stem cell like subtypes. Stem cell-like subtypes are resistant to therapy. To classify non-stem cell like subtypes, other immunohistochemistry stains are needed Vimentin should be included in immunohistochemistry staining panels because EMT is correlated with the invasion of breast cancer cell and mesenchymal character. The importance of this study was to identify of stem-cell-ness/stemness which will influence therapy.

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