

Case Study: Heterogeneity of and CD44+/CD24- Cancer Stem Cell Subpopulation of Breast Cancer Patients in Bandung, Indonesia

Wahyu Widowati^{1,*}, Diana Krisanti Jasaputra¹, Yusuf Heriady^{2,3}, Roro Wahyudianingsih¹, Dian Ratih Laksmiawati⁴, Ahmad Faried^{5,6}, Hanna Sari Widya Kusuma⁷, Fadhilah Haifa Zahiroh⁷, Rizal Rizal^{7,8}, Chrismis Novalinda Ginting⁹ and I Nyoman Ehrich Lister⁹

¹Faculty of Medicine, Maranatha Christian University, West Java 40164, Indonesia

²Faculty of Medicine, Bandung Islamic University, West Java 40116, Indonesia

³Surgery Department, Al Ihsan Hospital, West Java 40381, Indonesia

⁴Faculty of Pharmacy, Pancasila University, Jakarta 12640, Indonesia

⁵Departement of Neurosurgery, Faculty of Medicine, Padjadjaran University, Bandung, West Java 40161, Indonesia

⁶Oncology & Stem Cell Working Group, Dr. Hasan Sadikin Hospital, Bandung, West Java 40161, Indonesia

⁷Biomolecular and Biomedical Research Center, Aretha Medika Utama, West Java 40163, Indonesia

⁸Biomedical Engineering, Department of Electrical Engineering, Faculty of Engineering, Universitas Indonesia, West Java 16424, Indonesia

⁹Faculty of Medicine, Universitas Prima Indonesia, North Sumatera 20118, Indonesia

(*Corresponding author's e-mail: wahyu_w60@yahoo.com, wahyu.widowati@maranatha.edu)

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Abstract

Breast cancer (BC) cells characteristics have played a crucial role in clinical strategies decision-making and patient outcome prediction. Although there have been several studies examining this, there are still relatively few that analyze stem cell subpopulations or protein biomarkers. The conducted research sought to elucidate breast cancer subtype characteristics based on immunohistochemical analysis and cancer stem cell presence based on flow cytometry analysis in three breast cancer samples from Bandung, Indonesia. A flow cytometry analysis was conducted to determine how much CD44/CD24 was expressed as a marker of cancer stem cell in isolated BC cells using various cell references, such as cell lines of SKBR-3, MDA-MB-468, and Hs587T. Immunohistochemistry analysis was carried out to investigate the expression of p53, HER2, PGR, Ki67, and ER in sample tissue sections. According to histological analysis, 2 samples were solid mammary carcinoma, and 1 sample was mammary fibroadenoma. Immunohistochemistry and histological analysis showed that all BC tissues are classified as Luminal B. In addition, a large percentage of CD44+/CD24- subpopulation was found in isolated BC cells (> 90 % in patients 1 and 3; > 60 % in patient 2). All samples showed similarity to Hs587T cell line characteristic. This study has successfully characterized solid mammary tumors and mammary fibroadenoma with luminal B characteristics. All specimens contained a high proportion of cancer stem cells.

Keywords: Breast cancer, CD44+/CD24-, Cell line, Histological analysis, Hs587T, Immunohistochemistry, Immunophenotyping

Introduction

Breast cancer (BC) is a worldwide health issue that contributes to many cancer deaths in women [1]. There are limitations in the preliminary detection of BC, even though mortality and incidence of cancer, especially breast cancer, increased within the last two years [2]. In 2012, BC contributed to 23 % of all cancer deaths, pursuant to the World Health Organization (WHO). The same report also shows that 1 in 3 women in Asia will develop BC during their lifetime [3]. BC is a heterogeneous disease formed up of several entities exhibiting various behaviors [2]. High diversity has been discovered in recent molecular characterization within and between tumors and BC [4]. It is still difficult to get incremental information about this disease treatment [5]. A mammary tumor consists of a mixture of malignant and non-malignant cells. In heterogenous cells, several molecular processes take place that are related to various clinical outcomes [6].

Genetic evolution has led to the production of breast cancer sub-clonal heterogeneity. Predicate on the grade and kinds of histology, BC can be divided into clinically and biologically meaningful subgroups. The proliferative activity (measured by the mitotic index), aggressiveness, and degree of proliferation are all indicated by the BC grade. Histological type refers to the morphological and cytological patterns of the tumors. The histopathology of BC, which arises from distinct microanatomical structures of BC, is related to the invasiveness of the tumor [7]. Based only on histomorphology and development patterns, the WHO identified 21 distinct histological kinds of breast cancer [8]. Cancer's histopathology in breasts defines subgroups with regard to the prognosis for particular forms of breast cancer. However, histological type alone only gives information about different biological features and crucial clinical implications, not on BC therapy [7].

Protein biomarkers provide fundamental diagnostics for BC aggressiveness and forecast treatment responses [9]. Histological stratification, distinguished by the presence of estrogen receptor (ER), ERBB2 (Erythroblastic Oncogene B), human epidermal growth factor receptor (HER2) and progesterone receptor (PR), lays the foundation of breast cancer classification [10]. According to this stratification, BC is classified into 3 subgroups: 1) hormone-positive BC, which represents 70 % of BC cases, is marked by ER and/or PR overexpression and has a better prognosis than the other 2 subtypes; 2) HER2+, which represents 20 % of BC cases, is defined by human epidermal growth factor receptor 2 (HER2) amplification and is more aggressive than the former; and 3) triple-negative BC (TNBC), which accounts for 15 - 20 % of BC cases, lacks those 3 receptor expression and is recognized for its aggressive course and poor prognosis [11]. The aforementioned assessment is mandatory in all BC patients for forecasting response to endocrine therapy and anti-HER2 therapy in patients with initial or developed stages [12]. Another important biomarker is Ki67, a nuclear protein associated with cellular proliferation that correlates with tumor size and grade. Higher expression (> 35 %) of Ki67 has been associated to an elevated risk of demise. Ki67 was notably higher in ductal TNBC [13]. Ki67 has also been a predictor for neoadjuvant and adjuvant therapy [12].

Further molecular analysis based on 50 internal genes (PAM50) has resulted in the classification of four major subtypes of BC: luminal, basal-like, Her2, and normal-like. Two main categories of breast cancer tumor types - ER positive and ER negative - were established. The breast luminal cells' genes, such as GATA-binding protein 3, X-box binding protein 1, and Estrogen Receptor 1, are expressed comparatively highly in the ER+ tumors. These tumors are further characterized using particular histological staining, which reveals that none of them express Erb-B2 (HER-2) at high levels and that they are positively stained with luminal cell keratins 8/18. This group is later known as the luminal group. The ER- group is later divided into four different subgroups. One subgroup expresses characteristics of breast basal epithelial cells with low expression of ER. This group is stained positive for keratin 5/6 and 17, later known as the 'basal-like' type. Another group overexpresses the Erb-B2 oncogene with sharing characteristics with basal-like tumors, later known as the 'HER-2 type'. The 'normal-like' type expresses 'normal breast' gene expression and basal epithelial cells, adipose cells gene characteristics with low luminal epithelial cells characters [14]. Further analysis based on IHC markers of ER/PR/HER-2 and Ki67, divides the group into luminal A and luminal B groups. Luminal A is defined as ER+/PR+/HER2- and Ki67low. While luminal B is either ER+/PR-/HER2- or ER+/PR+/HER2-, Ki67high or ER+/PR+ or -/HER2+ with Ki67high/low. Overexpression of HER2 is determined as ER-/PR-/HER2+, and basal-like as ER-/PR-/HER2- (triple negative) [15].

Cancer stem cells (CSCs) are acknowledged as the source of functional intra tumoral heterogeneity in terms of initiating capacity. The CSCs display stem/progenitor cell characteristics, while also providing plasticity, which leads to tumors, including cells that correspond to multiple development levels of the normal mammary gland hierarchy. Therefore, all cell types within the tumor's hierarchical structure must be taken into consideration while developing treatments. Based on the expression of ALDH+, CD133+, CD44hi/CD24-, or CD29hi/CD61+, several investigations have demonstrated the presence of overlapping different CSC populations, which reflects "mesenchymal-oriented CSCs" and "epithelial-like breast CSCs" [16].

One of the key strategies to control cancer, which contributes to 50 % of cases of human cancer, is the product of p53, the tumor suppressor gene in humans. This suppressor gene induces cell death through apoptosis, stimulates the transcription of p16 and p21 as other tumor suppressors, and promotes DNA repair [4,17]. The TP53 mutation has been known to be correlated with poor chemo/hormone/radiotherapy responses. TP53 status screening could be useful for predicting BC prognosis and choosing treatment for breast cancer [18].

Moreover, these classifications and markers play a significant role in clinical strategy, decision-making, and patient outcome prediction [19]. Thus, determining the patient's characteristics after being

diagnosed with BC is necessary in the interest of choosing the correct treatment and achieving the highest level of pharmaceutical effectiveness. The breast cancer characterization in Indonesia, however, hasn't been well reported. In previous research, Siregar and Christopher [20] reported that young women suffering from breast cancer have a tendency to get aggressive biological characteristics' tumors with no special type of invasive carcinoma. Moreover, Widodo *et al.* [21], focused on clinicopathological characteristics in Triple Negative Breast Cancer. The study reported that TNBC tends to occur in young patients, marked by tumors of substantial size, metastatic lymph nodes, and a high histopathological grade. It is necessary to characterize breast cancer cells in more depth since this information might aid Indonesia develop novel approaches to treating breast cancer. Therefore, this research elucidated breast cancer subtypes characteristics from Al-Ihsan Hospital, Bandung, Indonesia, based on the CD44/CD24 subpopulation and the expression of p53, ER α , HER2, PGR, and Ki67.

Materials and methods

Sample preparation

This study attained Ethical Committee approval from the Faculty of Medicine, Maranatha Christian University, and Immanuel Hospital, Bandung, Indonesia, with approval number 432/VII/S.Kep./FK-UKM/2017. There were 3 participants whose BC tissue was obtained: Patient 1 (P1) is a 41-year-old woman whose tumor section was acquired by biopsy; Patient 2 (P2) is a 22-year-old woman whose tumor section was attained through biopsy; Patient 3 (P3) is a 43-year-old woman, and a mastectomy was used to obtain her tumor section. All participants had signed the informed consent form. The specimens were immediately treated with 1 % Gentamicin (Gibco, 15750060), 1 % Antibiotic-Antimycotic (Ab-Am) (Biowest, L0010-100), 1 % Amphotericin B (Amp B) and Nanomycopulitine (Biowest, LX16-100) addition after being washed with PBS (phosphate buffer saline) 1 \times (Biowest X0515-500). The same media was used to transfer the specimens for further processing [20].

Histopathological and immunohistochemistry staining of breast cancer tissues

The BC subtype was determined from its morphology through hematoxylin and eosin staining and protein biomarker expression (HER2, ER, PGR, Ki67, and p53) through immunohistochemistry (IHC). BCs were preserved in 10 % neutral buffered formalin, embedded in paraffin blocks, and cut into 4 μ m thick sections. Sections were then transferred to glass slides and deparaffinized with xylene, then rehydrated in an ethanol serial dilution. Endogenous peroxidase activity blocking was done by the slides fresh 0.3 % hydrogen peroxide incubation at room temperature for 15 min in methanol. Antigen retrieval was then performed at 121 $^{\circ}$ C in pH 6.0 10 mM citrate buffer for 10 min. Normal serum was later blocked through incubation in 5 % skimmed milk at room temperature for 30 min. In 1 % bovine serum albumin-contained PBS, the specimens were stained with eosin or the primary p53 polyclonal antibody (ElabSci, E-AB-32468), ER α polyclonal antibody (ElabSci, E-AB-31381), HER2 polyclonal antibody (ElabSci, E-AB-32197), PGR polyclonal antibody (ElabSci, E-AB-32651) and Ki67 polyclonal antibody (ElabSci, E-AB-63523) at 1:200, 1:100, 1:100, 1:100 and 1:200 dilution, respectively at 4 $^{\circ}$ C overnight. Then, slides were washed and for the next 30 min, secondary antibody (Histofine SAB-PO (M), Nichirei) was added at room temperature. Haematoxylin was used as a light counterstain on the specimens. Since there was no observable staining, each primary antibody was replaced with normal mouse IgG to create negative controls. The proportion (%) and intensity of expression were used to score the protein expression [20-22]. Protein expression scoring is assigned (-) if there is no expression, (+) if the expression is weak, (++) if the expression is moderate, and (+++) if the expression is strong.

Breast cancer cells isolation

PBS was used to wash cancer tissues on a petri dish 1-fold. Then, followed by cutting them into the size of 1 - 2 mm. Cancer tissues were digested with Trypsin 0.1 % (Biowest, L0931-500) enzymes, 1 mg/mL Hyaluronidase (Sigma, 515397), 4 mg/mL Collagenase Type I (Gibco, 10114532) and homogenized inside shaker incubator (300 rpm, 37 $^{\circ}$ C, 5 % CO $_2$) for 3 h, subsequently continued with overnight incubation. Using a 70 μ m cell strainer (Corning, 431752), the sample was filtered before being centrifuged for 10 min at 1,600 rpm (MPW260-R). Centrifugation of the supernatant-removed pellet was done 2-fold for 5 min at 1,600 rpm each after being washed with PBS 1 \times . Then, the whole growth medium (DMEM-F12 (Biowest, L0093-500), Amp B, 1 % Ab-Am, 0.1 % Gentamicin, and 1 % Nanomycopulitine supplemented with 10 % FBS (Biowest, S1810-5000)) was then resuspended to the pellet and cultured at 37 $^{\circ}$ C with 5 % CO $_2$ [20,22-24].

Cancer stem cells characterization in isolated breast cancer cells

Flow cytometry was used to characterize passage 3 of the cultured breast cancer cells (Macsqunt, Analyzer 10) at 1×10^5 cells per tube density. Based on manufacturer instructions, PE labeled CD24 (Biolegend, 311118) and FITC labeled CD44 (Biolegend, 338804) were used to stain the cells. Surface marker detection was performed in triplicate. The 18 Hs587T (ATCC®HTB-126), SKBR-3 (ATCC®HTB-30) and MDA-MB-468 (ATCC®HTB-132) cell lines were used as comparisons.

Results and discussion

Determination of breast cancer subtype using histopathology analysis

There is still a need for the isolation and characterization of BC cells, particularly in Indonesia, as no research has been done on the characterization and analysis of breast cancer cells subtype from Indonesian patients. In addition, the significance of the finding is increased by the absence of an effective cell model for breast cancer medication. Breast cancer cell lines play a crucial role in basic research. However, they didn't always accurately reflect the heterogeneity of breast cancer [20].

Table 1 displayed 3 BC patients' characteristics. Three BC samples that were suspected of having histopathology revealed 2 different types of malignancy. Patients 1 and 3's samples were more malignant and consisted of solid tumor cells. All tumor cells had polygonal or epithelial morphology and eosinophilic cytoplasm. This tumor section was classified as mammary solid carcinoma. On the other hand, patient 2's sample contained less malignancy, and an element of the mass was made up of a ductular structure restrained by 1 to 2 epithelial cell layers organized in a lobus with fibrous hyperplasia intralobular tissue; no tumor cells were found in the tissue slice, and it was classified as mammary fibroadenoma (**Figure 1**). All determinations referred to WHO classifications of tumors of the breast [25]. Based on **Figure 2**, the isolated breast cancer morphology exhibited fibroblast-like cells and showed adherence to the plastic disk.

Table 1 Baseline characteristics of breast cancer patients.

Characteristics	Patient 1	Patient 2	Patient 3
Gender	Female	Female	Female
Age (years)	41	22	43
Tumor retrieval method	Biopsy	Biopsy	Mastectomy

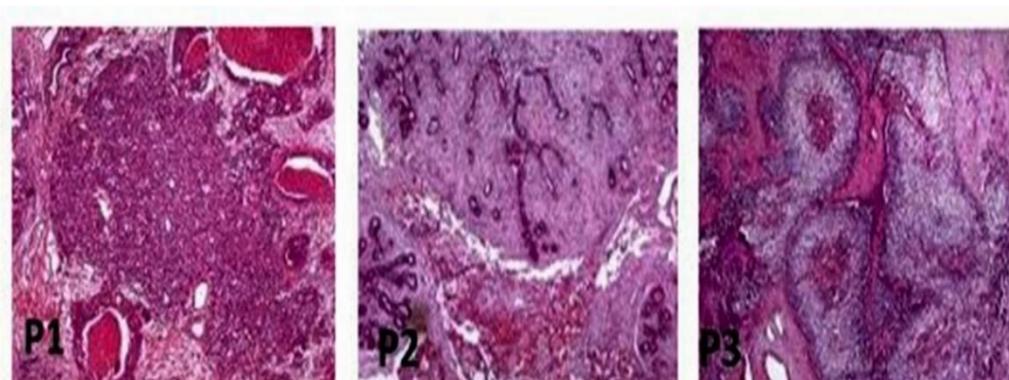


Figure 1 Examination of suspected BC samples using histopathology. Three samples revealed two distinct types. P2 was classified as a mammary fibroadenoma type, while P1 and P3 were classified as mammary solid types.

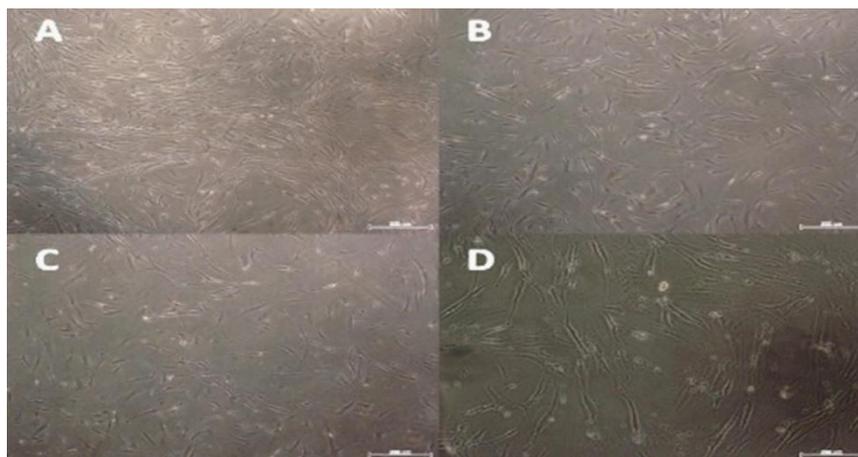


Figure 2 Morphological looks of isolated BC cells from 3 distinct patients showed fibroblast like cell. (A) P1, (B) P2, (C) P3 in magnification (40×), and (D) P3 magnification in (100×).

ER, PGR, Ki67, p53, and HER2 expressions in the isolated breast cancer

For the current investigation, BC from three different patients from Bandung, Indonesia, was isolated. Immunohistochemical analysis demonstrated that sample 1 strongly expressed ER, but weakly expressed HER2 and PGR. TP53 was not found expressed in this specimen. Sample 2 strongly expressed ER, PGR, but weakly expressed HER2. TP53 was weakly expressed in this specimen. Meanwhile, sample 3 showed strong expression for ER, PGR, HER2, and p53. All specimens showed moderate to strong expression of Ki67 (Figure 3), (Table 2).

Table 2 Qualitative data on protein expression in suspected BC samples.

Sample no.	HER2	p53	Ki67	PGR	ER
P1	+	–	++	+	+++
P2	+	+	+++	+++	+++
P3	+++	+++	+++	+++	+++

Note: (–) no expression; (+) weak; (++) moderate; (+++) strong expression.

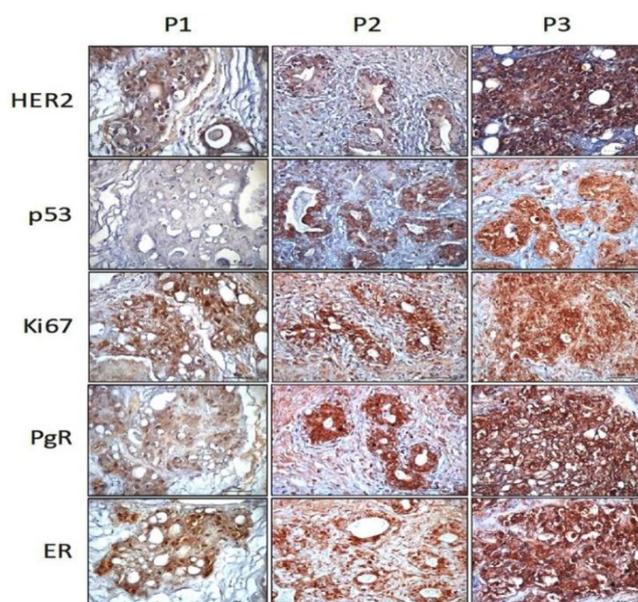


Figure 3 The results of the IHC analysis of isolated BC to assess HER2, p53, Ki67, PGR, and ER expression.

Referring to the IHC analysis, the 3 samples were classified as luminal B as all samples expressed ER+/PGR-/HER2- (sample 1), ER+/PGR+/HER2- (sample 2) and ER+/PGR+/HER2+ (sample 3) with Ki67 high in all samples [15]. In normal cells, p53 will be undetectable under IHC staining due to its short half-life. Contrarily, p53 in tumor cells will pile up in the nucleus on account of increased half-life and conformational structure alteration. The p53 protein was found to be high in sample 3, weakly positive in sample 2 and negative in sample 1.

However, this finding is not linear with the histological analysis that shows both samples 1 and 3 have higher malignancy compared to sample 2, which indicates that p53 mutation should be higher in sample 1 and 3 compared to 2. IHC analysis for p53 detection is not the best method, as this method could give false positive or false negative results. False positive results are caused by the stabilization of wild-type p53 protein due to cellular stress, while false negatives are generated by codon stop, frameshift, or destabilizing mutations. Identification of p53 IHC positivity did not always reflect a p53 mutation. The FASAY test could be used as an alternative method to detect TP53 status. Its status is indicated by the color change of transfected yeast with extracted mRNA from BCs [16]. However, IHC analysis indicated that not all recovered BC cells belonged to the basal cell type. This result was due to no correlation between CD44 and CD24 expression to ER, PGR, and Ki67 expression [19].

CD44+/CD24- subpopulation

Consequently, the majority of the isolated BC samples were likely BC stem cells. In established cancer cell lines, the population of CD44+/CD24- was also evaluated by flow cytometry. The outcomes of the flow cytometry test supported earlier findings that Hs587T was mostly CD44+/CD24-, SKBR-3 was CD44-/CD24+, and MDA-MB-468 was CD44+/CD24+. Thus, isolated BC cells shared Hs587T's cell surface marker phenotype (Figure 4).

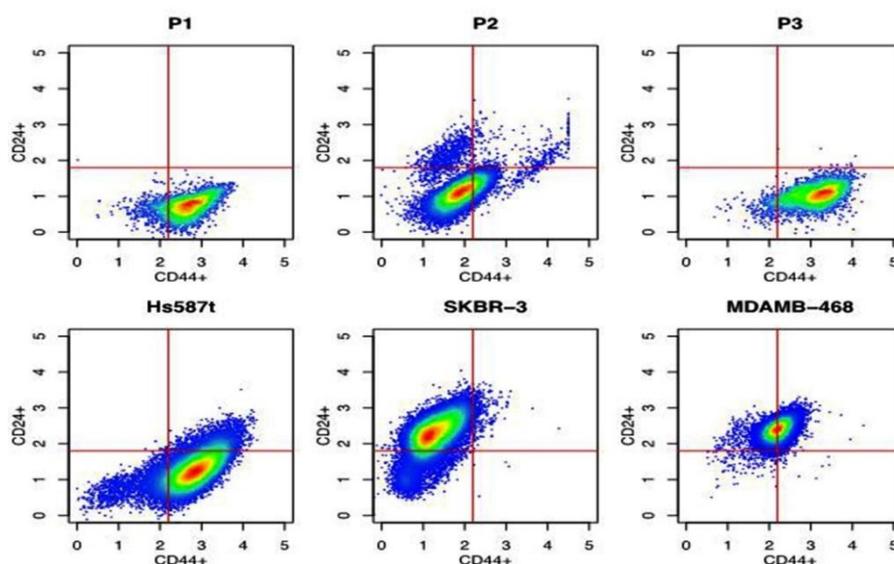


Figure 4 Percentage of CD44/CD24 expression as determined by flow cytometry in isolated BC cells. P2 has the lowest number of CD44+ cells, while CD24+ cells in P2 exhibited the highest amid the three samples in magnification (100×).

In isolated BC samples, we assessed CD44 and CD24 surface markers to investigate the cancer stem cell subpopulation. Isolated BC cells have an elevated amount of the CD44+/CD24- subpopulation, according to flow cytometry analysis (FACS). The highest subpopulation of CD44+/CD24- was obtained by P3 (95.65 ± 1.90 , $n = 3$) while P2 had the lowest (61.05 ± 1.60 , $n = 3$) (Table 3). All cancer types were successfully identified from this investigation using CD44+/CD24-. It had been noted that stem/progenitor cell characteristics were CD44+/CD24-. The lowest subpopulation of CD44+/CD24- was Patient 2 (normal-stem cells), Patient 1 (Luminal B) and Patient 3 (Luminal B), respectively. We used Hs587T (triple negative), MDA-MB-468 (triple negative), for the comparison. The results revealed that all of the compared cells' expression patterns were consistent with those of earlier research; Hs587T was predominantly CD44+/CD24-, MDA-MB-468 was CD44+/CD24+, and SKBR-3 was CD44-/CD24+ [26].

Table 3 The characteristics of isolated BC cells and reference cells based on CD44 and CD24 expression.

Cell type	CD44/CD24 Immunophenotyping			
	CD44+/CD24-	CD44+/CD24+	CD44-/CD24+	CD44-/CD24-
Hs587T	87.85 ± 0.8	8.12 ± 1.01	0	4.03 ± 0.21
SKBR-3	0.13 ± 0.02	9.09 ± 1.26	81.68 ± 1.53	9.09 ± 1.69
MDAMB-468	0.38 ± 0.07	97.12 ± 0.19	1.90 ± 0.04	0.61 ± 0.09
Patient 1	87.85 ± 0.80	8.12 ± 1.01	0	5.44 ± 1.16
Patient 2	61.05 ± 1.60	3.16 ± 0.30	2.47 ± 0.13	33.32 ± 2.00
Patient 3	95.65 ± 1.90	0.21 ± 0.10	0	4.14 ± 1.79

All samples had characteristics that were similar to those of the Hs587T cell line and wholly distinct from those of the SKBR-3 and MDA-MB-468 cell lines, according to a multidimensional scale analysis. Compared to other samples, P2 revealed a different character, while P1 and P3 among the 3 samples exhibited a high correlation (**Figure 5**). Hs587T belonged to basal mesenchymal cells. In addition, the other cell lines that are comparable to isolated BC cells' CD44/CD24 expression are TMD-436 (basal myoepithelial), SUM1315 (basal) and MDA-MB-231 (basal mesenchymal). Cancer cells are known to become more invasive when their CD44+/CD24- expression is increased. Due to the fact that distant metastasis was associated with an increased prevalence of CD44+/CD24- but not other clinical parameters [24]. Recent research showed that CD44 can boost the effectiveness of breast cancer distant metastasis [27].

The results obtained from this research are comparable to prior studies, which CD44 and CD24 markers had a correlation with breast cancer tumorigenesis [28].

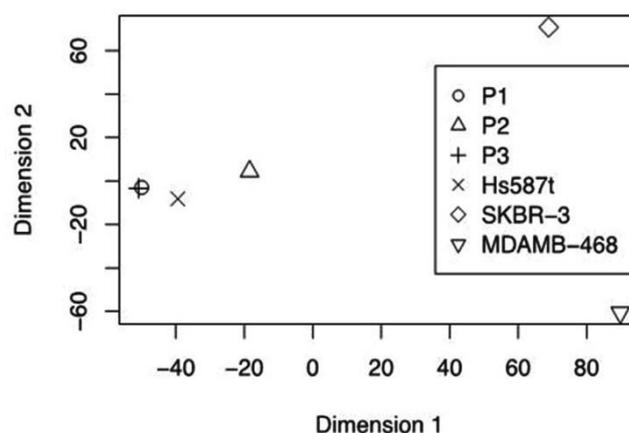


Figure 5 Multidimensional scale (MDS) analysis of three isolated BC cells (P1, P2 and P3) reference cell lines based on immunophenotyping of CD44/CD24 data. *All samples demonstrated notable relatedness to Hs587T, compared to the other two reference cell lines, SKBR-3 and MDA-MB-468.

This study effectively carried out methods to comprehend the characteristics of breast cancer (BC). However, it is restrained by some limitations, as we didn't have the opportunity to use more than three breast cancer samples and more surface markers to elucidate breast cancer subtype characteristics.

Conclusions

In summary, there are different CD44+/CD24- expression patterns shown by different cancer subtypes of BC cells isolated from three different patients. CD44+/CD24- was the highest subpopulation of isolated cells. Moreover, the solid mammary tumor and mammary fibroadenoma with luminal B tumor characteristics have been successfully characterized. All specimens contained a high proportion of cancer stem cells. This study obtained isolated BC cells characteristics by protein biomarkers. Characterization of BC cell protein expression could be beneficial for the selection of the BC treatment's target mechanism.

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