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ORIGINAL PAPER

Isolation, Characterization and Proliferation of Cancer Cells from Breast Cancer Patients

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ABSTRACT

Introduction: The most cases of cancer death, which are in the first ranks among cancers suffered by women is breast cancer. The breast cancer therapy for patients has been done, but still not optimal, so it is necessary to understand the mechanism of therapy in model cell of breast cancer. **Aim:** This study aims to develop an isolation technique of breast cancer cell from patients as a cancer cell model. **Material and Methods:** Breast cancer cell isolation is performed by enzymatic methods using the collagen I and hyaluronidase. Then, breast cancer cells were characterized using flow cytometry based on the CD44/CD24 expression where MDA-MB468 and MCF-7 breast cancer cell lines were used as positive controls. Estrogen receptor (ER), progesterone receptor (PR), p53, HER2, and Ki67 expression were assessed using an immunohistochemistry assay. **Result and Discussion:** The morphology of cancer cells was fibroblast like cells on the day 7th after isolation. Isolated breast cancer cells expressed 95.33±0.47% of $CD44^{+/}CD24^{+}$ and human epidermal growth factor receptor 2 (HER2) low expressions. Isolation of breast cancer cells can use In-house enzymatic protocol. Isolated breast cancer showed the same expression as MDA-MB468 ($CD44^{+/}CD24^{+}$) and $HER2^{-}$ compared to MCF-7 cell lines ($CD44^{-/}CD24^{+}$). **Conclusion:** These cells belonged to a basal type of breast carcinoma and expressed CD44+/CD24+, then isolated BCCs can be used as model cancer cells for further research.

Keywords: breast cancer, cell lines, CD44 antigen, CD24 antigen, enzymatic assay.

1. INTRODUCTION

Breast cancer is the first rank among cancers suffered by woman causing death. It often occurs recurrence after treatment (1). In the Asia-Pacific, nearly a quarter of the population of women were diagnosed with breast cancer (24%), with the largest percentage occurring in China (46%), Japan (14%), and Indonesia (12%). The type of cancer in Indonesia is dominated by breast cancer (30%) and cervical cancer (24%) (2).

Breast cancer classification was based on different parameter, i.e.: tumor grade, lymph node status, histological type, the presence of estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). Molecular profiling proved that breast cancer could be classified into: normal, basal, luminal A, luminal B and HER2 based on ERα, progesterone receptor (PR) and HER2 expression in immunohistochemical assay (3). MDA-MB453 is $HER2^{+}$ type which expresses high Ki67 (4), while MDA-MB-468 is a basal type with ER, PR^{-} and $HER2^{-}$ expression and MCF7 is luminal A type with of ER^{+}, PR^{+} and $HER2^{-}$ expression (5).

Breast cancer treatment depends on the stadium of breast cancer. Therefore, the selection of therapy at each stadium of the cancer is different (6). Surgery and chemotherapy are types of cancer therapy, which is effective to treat breast cancer (7). But, surgical therapy is costly and high risk of infection during surgery, as well as patient complaints regarding cosmetics (8). Chemotherapy can destroy normal cells and cause side effects, including: neutropenia, anemia, thrombocytopenia, alopecia, mucositis (8). The cell line is the most important factor in studying therapeutics target, carcinogenesis, and signal pathways for breast cancer (9).

2. AIM

The cell line of study is based on its biological aspects. Hence, research needs to be done on breast cancer iso-

lation. Therefore, it is essential to have isolated breast cancer cells from breast cancer patient as a cancer cell model in order to determine the effective cancer therapy.

3. MATERIAL AND METHOD

Sample Preparation

The sample was the tissue from breast cancer patients 52 years old with ductal carcinoma mammae sinistra grade III and invasive to lymfovascular. The informed consent used the guidelines approved by the Institutional Ethics Committee collaboration between Maranatha Christian University, Bandung, Indonesia and Immanuel Hospital Bandung, Bandung, Indonesia. Tissue was then washed using phosphate buffer saline (PBS 1x, Gibco 1740576) and preserved in the transfer medium consist of PBS 1x + 1% Abam (Gibco, 1772653) + 1% Amphotericin B (Gibco, 15290026) + 0.1% Gentamicin (Gibco, 15750060) before being isolated (10).

Breast Cancer Cell Isolation

Medium preparation: Dulbecco's Modified Eagle Medium (DMEM, Gibco 11995065) : F-12K (Sigma, I6634) (1:1), 5% Fetal Bovine Serum (FBS, Gibco, 10270106), 1% Antibiotic-antimicotic (Gibco, 1772653), 1% Amphotericin B (Gibco, 15290026), 0,1% Gentamicin (Gibco, 15750060) were dissolved then filtered using Syringe Filter PES 0, 22µm (TPP, 99722). Enzyme preparation: Collagenase Type I (Gibco, 10114532) 4 mg/ml, hyaluronidase 1 mg/ml, Trypsin 0,1% (Gibco, 25200072) were dissolved in basal medium that consists of DMEM: F-12K (1:1), then filtered using syringe filter 0,22 µm PES (TPP, 99722).

Cells Isolation: Prepare a surgical device, PBS 1x and sterile petri dish. The tumor tissue was washed with PBS 1x on a petri dish and then cut it into small pieces using surgical scalpel. The sample fragments were washed again with PBS 1x (Gibco, 1740576), inserted into a 50ml falcon tube (TPP, 91050) containing the mixed enzyme solution and then incubated 16 hours, 300 rpm, 37 °C, 5% CO_2. After incubation sample was filtered using 100 µm cell strainer (Corning, 431752) then centrifuged at 1600 rpm for 10 minutes. Supernatant was discarded and the pellet was washed with PBS 1x and centrifuged at 1600 rpm for 5 minutes. The pellet was then resuspended with the complete growth medium. The cell suspension was next incubated at 37°C, 5% CO2 (modified method from 10).

Breast Cancer Cells Characterization

Breast Cancer Cells (BCCs) at P3 were characterized using flowcytometry (Macsquant, Analyzer 10) for positive and negative markers of breast cancer cells at density $1x10^5$ cells/tube. The cells were then stained with CD44 and CD24 according to manufacturer protocol (Biolegend kit, 338804/311118). The experiments and measurements of surface marker were performed in triplicate (11).

Proliferation Assay

BCC was cultured in medium consisting of DMEM: F-12K (1:1), 5% FBS, 1% Antibiotic-antimycotic, 1% Amphotericin B, 0,1% Gentamicin and the medium was replaced every 3 days. 3 x 10^5 cells were seeded on T25 flask and cultured in complete medium until reaching confluence 80% around 3 days for proliferation assay. Briefly, cultured cells was detached using trypsin (Gibco, 25200072), then incubated for 1-3 minutes at 37°C, complete medium was added to neutralize trypsin and the detached cells were centrifuged (MPW-2000) at 1600 rpm for 5 min at room temperature. The cell pellet was resuspended with trypan blue solution (Sigma, 25200072) and diluted in 1:1 dilution. Then, cells were counted using a haemocytometer (Neubauer, 17849). Population Doubling (PD) was counted at every passage with the formula:

$$PD = [\log_{10}(NH) - \log_{10}(NI)]/\log_{10}$$

NI is the inoculum cell number and NH is the cell harvest number. Then, to determine cumulative PD data by added the PD at the previous passage.

The PD time (PDT) was determined by the formula (11).

$$PD\ time = t\ (time)/PD\ (in\ days)$$

Immunohistochemistry (IHC) Analysis

Breast cancer cells were processed using anti-^{S727}p-STAT3 (BD Biosciences), anti-CA-IX and anti-EGFR (clone 31G7, Zymed Technologies, Invitrogen, Burlington, ON) protocols. The Idetect Ultra HRP Detection System (ID Labs Inc, London, Ontario) was used to visualize. Counterstaining was performed with Gill modified hematoxylin (Harleco®) (12).

Statistical Analysis

Statistical analysis was conducted using SPSS software (version 20.0). Data were presented as Mean ± Standard Deviation. Significant differences among treatments were determined using the one-way Analysis of variance (ANOVA) and $p < 0.05$ were considered as statistically significant, along with Turkey honestly significant difference post hoc test and 95% confidence interval. Z score values were used for plotting the protein expression based on immunohistochemistry signal. Clustering methods based UPGMA statistical analysis were performed by R software ver.3.4.3.

4. RESULTS

Isolation of BCCs with enzymatic method

BCCs were successfully isolated using an enzymatic method and cultured until passage 9. The cells attached to the surface of the plastic disc a day after seeding (P0) and were maintained up to two weeks. The cells showed short fibroblastic-like morphology in growth medium and there were still blood cells at the beginning of P0 (Figure 1A). From the first passage onwards, particularly at P1, P2, P3, the cells in

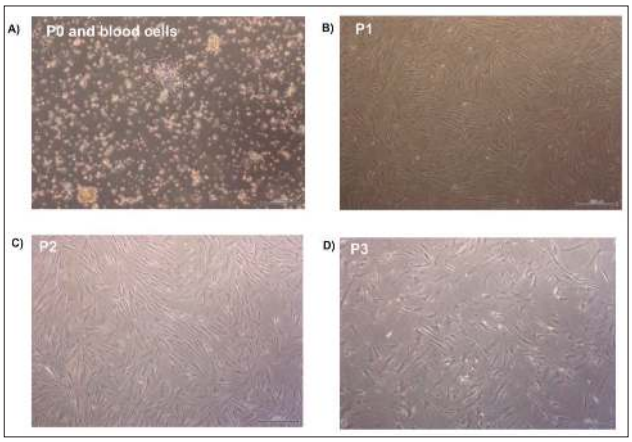

Figure 1. The morphology of breast cancer cells in the different passages. (A) BCCs at P0 and blood cells. (B) BCCs at P1. (C) BCCs at P2. (D) BCCs at P3. Magnification 40x. Scale Bars: 500uM.

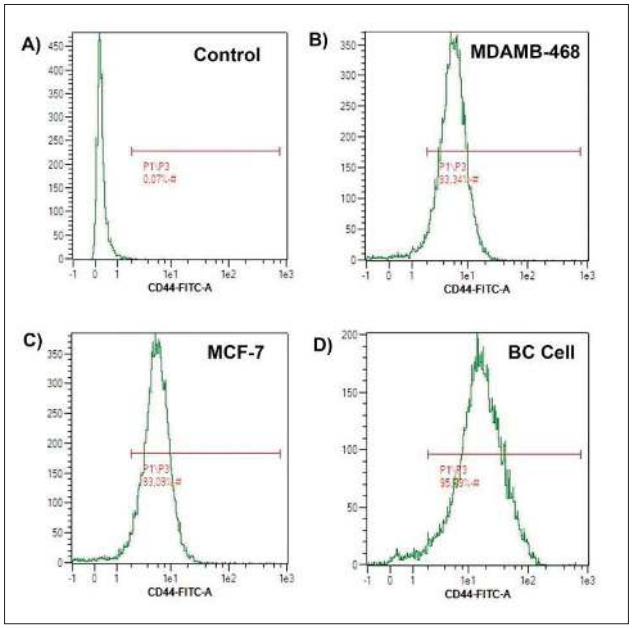

Figure 2. Density plot CD44 expressions in the unstained cell as a control (A), MDAMB-468 cell (B), MCF-7 cell (C), and breast cancer cell/BC cell (D)

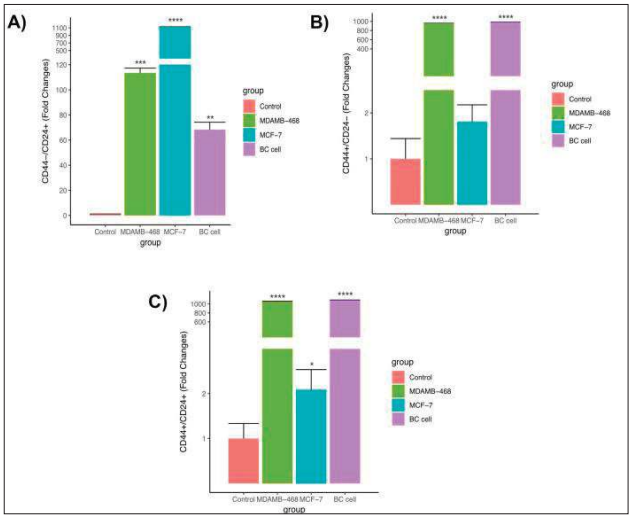

Figure 3. Expression of CD44-/CD24+ cell (A), CD44+/CD24- cell (B) and CD44+/CD24+ cell (C) characterization in breast cancer cell (BC cell) compared to MCF-7 cell lines and MDAMB-468 cell lines. Significance difference with p value p<0.05

growth medium changed their shape into long fibroblast cells (Figure 1B-1D).

Characteristic of Breast Cancer Cells

The flow cytometry analysis displayed the biological properties of BCCs, which was compared to MDA-MB468 and MCF-7 as cells control. BCCs showed a highest $CD44^+$ cell intensity expression compared to the MCF-7 cell line, but had no significant difference with MDA-MB468. The intensity of $CD44^+$ in most cells around 1e1 (log1) (Figure 2). However, MDA-MB468 and BCCs had a lower number of $CD44^-/CD24^+$ cells than MCF-7. On the other hand, $CD44^+/CD24^-$ and $CD44^+/CD24^+$ of BCCs and MDA-MB468 were higher than MCF-7 (Figure 3).

Population Doubling Time of Breast Cancer Cells

PDT and PD rate were used to evaluate the breast cancer cell proliferation capacity. PDT is the time needed by cell to divide into two cells. According to the results shown in

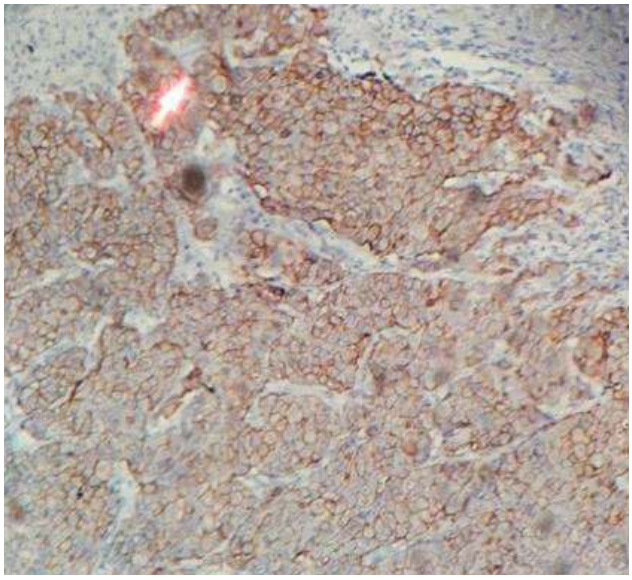

Figure 4. Immunoprofile of breast tissue from breast cancer patient. Immunohistochemistry result of HER2 expression in breast cancer.

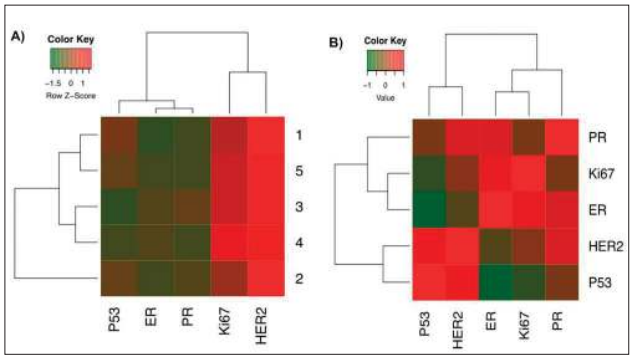

Figure 5. Gene Correlation Analysis (A) Heatmap protein expressions in breast cancer were analyzed by immunohistochemistry. (B) heatmap correlation graph by "Pearson" analysis in five surface protein expressions

Table 2, BCCs suggested high PDT at early P2 and P3 around 21.38±1.34 and 18.10±1.69 respectively compared to late passages (P7 and P8). It could be seen that BCCs reduced their PDT after every passaging (Table 1).

Immunohistochemistry (IHC) and Gene Correlation Analysis

IHC analysis was identified for immunoprofile of breast tissue from breast cancer patient (Figure 4). This procedure was performed to double check flow cytometry analysis of isolated BCCs in order to determine the molecular classification of cancer cell subtype. Five gene analysis (p53, ER, PR, Ki67, HER2) using an IHC staining method showed that HER2 was the strongest upregulated gene expression followed by Ki67 and other genes were down regulated (Figure 5A). In contrast, DeFazio-Eli et al. (2011) and Holliday and Speirs (2011) reported that MDA-MB468 suggested very low level of HER2. In a gene correlation analysis using Pearson method, HER2 had a high positive correlation with p53, PR, ER and low positive correlation with Ki67 (Figure 5B).

5. DISCUSSION

There are two kinds of breast cancer, namely ductal and lobular carcinoma that attack the milk duct and the milk producing gland respectively (13). Breast cancer therapy, which

has existed is still not optimal. Surgery therapy was costly and has a risk of infection during surgery (8). It also often occurs recurrence after treatment (1). Chemotherapy also has not shown optimal results, because the chemicals also target normal cells in the body, so that they can harm cells in the body. This causes new issues are alternative therapy is needed to determine the mechanism and causes of breast cancer. Therefore, to understand the mechanism in breast cancer cell, model cell is needed. Even though utilization of cell lines models cell for most the basic research, they are already engineered for some reasons which may not clinically related to the population of breast cancer.

The morphology of isolated BCCs at P0 show different shape compared to BCCs at P1 onwards. This is caused by the adaptation ability of isolated BCCs in a new environment. Since isolated BCCs have not only similar biological properties to MDA-MB468 but also its morphology like fibroblast cells (14). Isolated BCCs seem to keep with a good state in fibroblast like morphology at each passage.

This study uses MCF 7 and MDA-MB468 as a comparison because both cell lines are breast cancer cell lines, MCF7 is the luminal type with ER^+ and PR^+ and MDA-MB468 is the basal type with ER^-, PR^- and $HER2^-$ breast cancer cell line (5). IHC analysis suggests that isolated BCCs are the basal type of breast cancer cell. This finding highlights the importance of classifying isolated BCCs based on molecular classification using IHC assay and surface marker expression using flow cytometry analysis into consideration of clinical cancer therapy.

The isolated BCCs express low HER2 expression and high Ki67 expression based on IHC analysis. It is in line with research from DeFazio-Eli et al. that MDA-MB468 cell line has also very low levels of HER2. Also, Holliday and Speirs (3) mention that MDA-MB468 is a basal type because this cell line has ER^-, PR^- and $HER2^-$ and high Ki67 characteristic around 20%. Ki67 expression ranging under 50%, while cancer cell lines ranging from 20%-100% is one explanation of striking differences between cell lines and isolated primary tumor cell (9). The similarities between basal and triple-negative phenotypes cannot guarantee the type of breast cancer. In line with recent studies, there is still no convincing that triple negative is a feature of basal type cancer (15). Therefore, our finding can be assumed that isolated BCCs have the same characteristics as MDA-MB468 according to IHC assay and flow cytometry analysis.

According to the results shown in Table 2, breast cancer cell proliferation in the second passage need 21 days to doubling their population. While in the third and fourth passage need 18 day and 13 day to doubling their population respectively. In earlier passages, the cell takes time to proliferate because it adapts to the new environment, however, in the next passage the cell has adapted well and high proliferate. Furthermore, doubling time in cancer cells is faster than other cells isolated from human it is because cell cancer is a malignant cell that has a fast proliferation time than normal cells. It is supported by Liu et al. (16) she states that malignant cells have faster doubling times compared to other normal cells. The doubling time for MDA-MB468 (ATCC) should be 30-40 hours (17), while in this study PDT is 5,73 days in passage 8. This indicates that the isolated cells had a lower proliferation rate compared to MDA-MB468.

6. CONCLUSION

This study concludes that BCCs could express CD44+/CD24+, then expected act as model cancer cells effectively.

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LEMBAR HASIL PENILAIAN
SEJAWAT SEBIDANG atau *PEER REVIEW*

KARYA ILMIAH : JURNAL ILMIAH

Judul Karya Ilmiah (Artikel)	:	Isolation, Characterization and Proliferation of Cancer Cells from Breast Cancer Patients
Jumlah Penulis	:	12 orang
Nama-nama Penulis	:	**Wahyu Widowati,** Yusuf Heriady, Dian Ratih Laksmitawati, Diana Krisanti Jasaputra, Teresa Liliana Wargasetia, Rizal Rizal, Fajar Sukma Perdana, Annisa Amalia , Annisa Arlisyah, Zakiyatul Khoiriyah, Ahmad Faried, Mawar Subangkit
Status Penulis	:	Penulis Pertama ~~/ Penulis ke ....~~ / Penulis Korespondensi **)
Identitas Jurnal Ilmiah	: a.	Nama jurnal : Acta Informatica Medica
	b.	Nomor ISSN : 0353-8109
	c.	Vol., No., Bulan, Tahun : Vol.26, No.4, Dec 2018
	d.	Penerbit : Avicena Publishing
	e.	DOI Artikel (jika ada) : 10.5455/aim.2018.26.240-244
	f.	Alamat Web Jurnal : https://pubmed.ncbi.nlm.nih.gov/30692706/
	g.	Terindeks di : Scopus Q2, SJR 0.267

Kategori Publikasi Jurnal Ilmiah: *(beri tanda √ yang dipilih)*

- [√] ~~Jurnal Ilmiah Internasional~~ / Internasional Bereputasi **)
- [ ] Jurnal Ilmiah Nasional Terakreditasi
- [ ] Jurnal Ilmiah Nasional / Nasional terindeks ***)

HASIL PENILAIAN (*Peer Review*) :

No	Komponen Yang Dinilai		Nilai Maksimal JURNAL ILMIAH: Internasional / Bereputasi [√]	Nasional Terakreditasi [ ]	Nasional ***) [ ]	Nilai Akhir Yang Diperoleh *)
a.	Kelengkapan unsur isi karya	(10%)	4			3,4
b.	Ruang lingkup dan kedalaman pembahasan	(30%)	12			11,8
c.	Kecukupan dan kemutakhiran data/ informasi dan metodologi	(30%)	12			11,9
d.	Kelengkapan unsur dan kualitas penerbitan	(30%)	12			11,7
	Total	**100%**	**40**			34,8

Catatan Penilaian ARTIKEL oleh Reviewer :

a. Kelengkapan dan kesesuaian unsur.....
Unsur lengkap, hasil penelitian sesuai judul penelitian, mengikuti kaidah penelitian.

b. Ruang lingkup & kedalaman pembahasan .....
Data sangat memadai meliputi isolasi, karakterisasi kanker payudara yang diisolasi dari penderita kanker.

c. Kecukupan & kemutakhiran data serta metodologi..... Sumber pustaka mutakhir, menemukan metode isolasi & karakterisasi sel kanker payudara serta mampu berproliferasi dibanding

d. Kelengkapan unsur dan kualitas penerbit .....

Jurnal IJBMS terindeks scopus Q2 SJR 0,27. Penerbit Avicena

e. Indikasi plagiasi
Jurnal IJBMS terindeks scopus Q2 SJR 0,27. Penerbit Avicena tidak terindikasi plagiarism atau self-plagiarism. Dengan similarity index sebesar 17%

f. Kesesuaian bidang ilmu
Paper bidang biomedik sangat sesuai dengan bidang Ilmu penulis

REVIEWER 1

(Prof. Dr. Chrismis Novalinda Ginting, M.Kes)
NIK : 0115127801
UNIVERSITAS PRIMA INDONESIA

LEMBAR HASIL PENILAIAN
SEJAWAT SEBIDANG atau *PEER REVIEW*

KARYA ILMIAH : JURNAL ILMIAH

Judul Karya Ilmiah (Artikel) : Isolation, Characterization and Proliferation of Cancer Cells from Breast Cancer Patients

Jumlah Penulis : 12 orang

Nama-nama Penulis : **Wahyu Widowati,** Yusuf Heriady, Dian Ratih Laksmitawati, Diana Krisanti Jasaputra, Teresa Liliana Wargasetia, Rizal Rizal, Fajar Sukma Perdana, Annisa Amalia , Annisa Arlisyah, Zakiyatul Khoiriyah, Ahmad Faried, Mawar Subangkit

Status Penulis : Penulis Pertama ~~/ Penulis ke ....~~ / Penulis Korespondensi **)

Identitas Jurnal Ilmiah :

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- [√] ~~Jurnal Ilmiah Internasional~~ / Internasional Bereputasi **)
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- [ ] Jurnal Ilmiah Nasional / Nasional terindeks ***)

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No	Komponen Yang Dinilai		Nilai Maksimal JURNAL ILMIAH			Nilai Akhir Yang Diperoleh *)
			Internasional / Bereputasi [√]	Nasional Terakreditasi [ ]	Nasional ***) [ ]	
a.	Kelengkapan unsur isi karya	(10%)	4			3,6
b.	Ruang lingkup dan kedalaman pembahasan	(30%)	12			11,7
c.	Kecukupan dan kemutakhiran data/ informasi dan metodologi	(30%)	12			11,8
d.	Kelengkapan unsur dan kualitas penerbitan	(30%)	12			11,8
	Total	**100%**	**40**			38,9

Catatan Penilaian ARTIKEL oleh Reviewer :

a. Kelengkapan dan kesesuaian unsur
Jurnal ini sudah cukup lengkap dan ada kesesuaian antara unsur dan isinya

b. Ruang lingkup & kedalaman pembahasan
Ruang lingkup bahasan sudah memadai dan ada kedalaman dalam pembahasannya

c. Kecukupan & kemutakhiran data serta metodologi
Secara umum metodologi masih terbatas dan belum sepenuhnya

d. Kelengkapan unsur dan kualitas penerbit

Kualitas penerbit sudah memenuhi reputasi yang baik

e. Indikasi plagiasi

Secara umum belum terlihat adanya unsur plagiasi

f. Kesesuaian bidang ilmu

Jurnal ini sudah sesuai dengan bidang ilmu yang diteliti

REVIEWER 2

(Prof. Dr. Ermi Girsang, M. Kes)
NIK : 0117057501
UNIVERSITAS PRIMA INDONESIA

LEMBAR HASIL PENILAIAN
SEJAWAT SEBIDANG atau *PEER REVIEW*

KARYA ILMIAH : JURNAL ILMIAH

Judul Karya Ilmiah (Artikel)	:	Isolation, Characterization and Proliferation of Cancer Cells from Breast Cancer Patients
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Kategori Publikasi Jurnal Ilmiah: *(beri tanda √ yang dipilih)*

- [√] ~~Jurnal Ilmiah Internasional~~ / Internasional Bereputasi **)
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HASIL PENILAIAN (*Peer Review*) :

No	Komponen Yang Dinilai		Nilai Maksimal JURNAL ILMIAH: Internasional / Bereputasi [√]	Nasional Terakreditasi [ ]	Nasional ***) [ ]	Nilai Akhir Yang Diperoleh *)
a.	Kelengkapan unsur isi karya	(10%)	4			3,45
b.	Ruang lingkup dan kedalaman pembahasan	(30%)	12			11,65
c.	Kecukupan dan kemutakhiran data/ informasi dan metodologi	(30%)	12			11,75
d.	Kelengkapan unsur dan kualitas penerbitan	(30%)	12			11,85
	Total	**100%**	**40**			38,7

Catatan Penilaian ARTIKEL oleh Reviewer :

a. Kelengkapan dan kesesuaian unsur.....

Unsur lengkap, hasil penelitian sesuai judul penelitian, mengikuti kaidah penelitian.

Jurnal ini sudah cukup lengkap dan ada kesesuaian antara unsur dan isinya

b. Ruang lingkup & kedalaman pembahasan .....

Data sangat memadai meliputi isolasi, karakterisasi kanker payudara yang diisolasi dari penderita kanker

Ruang lingkup bahasan sudah memadai dan ada kedalaman dalam pembahasannya.

c. Kecukupan & kemutakhiran data serta metodologi

sumber pustaka mutakhir, menemukan metode isolasi & karakterisasi sel kanker payudara serta mampu berproliferasi dibanding

Secara umum metodologi masih terbatas dan belum sepenuhnya

d. Kelengkapan unsur dan kualitas penerbit

Jurnal IJBMS terindeks scopus Q2 SJR 0,27. Penerbit Avicena

Kualitas penerbit sudah memenuhi reputasi yang baik.

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f. Kesesuaian bidang ilmu

Paper bidang biomedik sangat sesuai dengan bidang ilmu penulis

Jurnal ini sudah sesuai dengan bidang ilmu yang ditekuni

Medan,
Reviewer 2

(Prof. Dr. Ermi Girsang, M.Kes)
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Medan,
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(Prof. Dr. Chrismis Novalinda Ginting, M.Kes)
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