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Stimulation of Allogenic lymphocytes by Dendritic Cells Derived from Human Umbilical Cord Blood Fused with Breast Cancer Stem Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Author PVP carried out the studies including primary culture, isolation of breast cancer stem cells, flow cytometry analysis. Authors STN and VQP performed the dendritic cell culture, fusion of dendritic cells and breast cancer stem cells. Author NKP participated designing the study and drafted the manuscript in cooperation with all other authors. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Cancer stem cells (CSCs) are cancer cells that possess characteristics associated with normal stem cells. CSCs represent a minor subset of cells in the tumor and are thought to be the reason for the initiation of disease, resistance to cancer treatment, and the occurrence of metastasis. Therefore, breast cancer stem cells (BCSCs) targeting therapies are considered as the promising therapy for breast cancer treatment. This research aims to evaluate the tumor associated antigens presentation of dendritic cells fused with breast cancer stem cells in dendritic cells based therapies.
Methodology: Human breast cancer stem cells are isolated from malignant breast tumors by enrich culture and fluorescent activated cell sorting. Human dendritic cells are isolated from umbilical cord blood by culturing CD14 monocytes in induced medium. Electrocution is used to fuse breast cancer stem cells and dendritic cells. Fusion cells are used to evaluate functions of dendritic cells (DCs) and also to stimulate T cells.

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Results: These findings indicate that fusion cells have the ability to present the antigens of breast cancer stem cell to T-cells, and regarding functionality.
Conclusion: They appear to be very good candidates for antitumor vaccine in breast cancer.

Keywords: Antitumor vaccine; breast cancer stem cell; cell fusion; dendritic cell; tumor-associated antigens; T-cell proliferation.

1. INTRODUCTION

Breast cancer is the most common disease and the leading cause of death in women. According to The International Agency for Research on Cancer in 1998, breast cancer accounts for 21% of all types of cancer in women all over the world. According to estimates of lifetime risk by the U.S. National Cancer Institute, about 13.2% of women in the U.S. will develop breast cancer. In Vietnam, breast cancer incidence among women is 53 per 100,000 women, compared to 145 per 100,000 for Caucasian women [26]. Breast cancer is becoming popular in Vietnam, but there are few studies on treatment of breast cancer for Vietnamese women until now. The cancer stem cell compartment is thought to be the reason for initiation of disease, resistance to treatment, and occurrence of metastatic disease. Cancer stem cells exist in the tumor burden with just around 1 to 2% of the total tumor burden, and are distinct from the more differentiated tumor cells [1, 5, 8, 9 ,14, 31, 35]. Until now, more than 30 new anticancer drugs have been introduced, but just a few survivals have been recorded for the various forms of cancer [40], though the cancer stem cell population are thought to have the ability to escape from treatments [7]. The theory suggests that traditional antitumor methods eliminate the normal cancer cells which compose a large part of the tumor, but cannot touch the cancer stem cells (CSCs). This remaining CSCs population has the ability to form a new tumor, resulting in tumor relapse. Therefore, finding and eliminating these CSCs is essential, and is becoming a central goal of cancer therapy. Cancer immunotherapy is one of the good ways to the pursuit of this purpose.

Cancer immunotherapy is a method that enhances the immunology system to identify and fight cancer. One of the popular cancer immunotherapies is cancer vaccine. Similar to traditional vaccines such as measles or polio vaccine, cancer vaccine bases on the antigen that is carried by cancer cells, and facilitates the immune system to recognize it as a foreigner and kill them in the next encounter. Because of the ability of dendritic cell (DC) for antigen presenting, using DCs for enhancing antitumor responses is an excellent choice in cancer vaccine researches. Most studies in finding out the way to induce antitumor effect in designing cancer vaccine involve DC [2]. Until now, there are many methods to load tumor associated antigen DCs, such as pulsing DCs with synthetic peptides derived from the known TAAs, tumor cell lysates, apoptotic tumor cells, and tumor RNAs [2,4,6,10,20,25, 27,36]. However, these strategies still have some limitations because of a limited number of known antigenic peptides and down-regulation of antigens for hiding from the immunological defense [3,28,32,37]. Moreover, the results of clinical trials showed that just a small number of patients give responses when using DCs pulsed with antigen-specific peptides [24,37].

To avoid this problem, it is necessary to target either multiple antigens or essential antigens, with an approach that has been developed by fusing DCs with tumor cells [10]. In this way, a set of antigens including unknown antigens can be presented to the immune system by MHC I and II molecules of DCs [10,12,13,21,19,18]. The fusion of DC and tumor cell is conducted

through chemical, physical, or biological means resulting in fusion cells which combine co-stimulatory molecules, efficient antigen-processing and presentation machinery of DCs, and the abundance of other antigens including those yet to be unidentified from tumor. In the fusion cells, the antigen of tumor cells is demonstrated to be processed by the endogenous machinery of DCs. This mechanism gives the DCs/tumor vaccine an advantage in comparison with the whole tumor lysate because endogenously processed antigen is easily accessed by MHC class I pathway [17,34].

With the aim to establish effective DC vaccine to treat breast cancer, we used the Vietnamese breast cancer stem cells line (VNBRCA) to fuse the dendritic cells, preliminary with the purpose of characterizing the fusion cells (FCs), and test the ability of presenting the tumor-associated antigen to T-cells.

2. MATERIALS AND METHODS

2.1 Collecting Umbilical Cord Blood

Fetal cord blood was collected from a healthy pregnant woman with agreement between the Hospital, donor, and laboratory. After giving birth, blood was taken from fetal cord and treated with anti-coagulant CDPA (Terumo, Japan).

2.2 Monocytes Isolation and Differentiation of Dendritic Cells (DCs)

Monocytes were isolated from fetal cord blood by density-gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Sweden), and were then cultured in IMDM (Sigma-Aldrich, USA) medium, in which 10% Fetal Bovine Serum (FBS) was added to it at 37ºC for 2 to 3 h. CD8+ T cells in the supernatant fraction were isolated by flow cytometry. The adherent-cells were cultured to differentiate the DCs as the following: monocytes were cultured for 9 days in RPMI 1640 medium (Roswell Park Memorial Institute), and 40 ng/ml interleukin-4 (IL-4), 50 ng/ml GM-CSF (Santa-Cruz, USA), and 10% FBS were added to the medium. A fresh medium to which cytokines were added was additionally provided at a 3-day interval to provide nutrient.

2.3 Characterization of Dendritic Cells

Before and after induction, marker surface of DCs were analyzed by flow cytometry to determine whether DCs is successfully differentiated or not. There were some antibodies used such as HLA-DQ, HLA-DR, CD40, CD80 and CD86 (BD Pharmingen). DCs were incubated in 100 µl PBS containing 5 µl of each monoclonal antibody for 20 min in the dark at room temperature. Cells were then washed three times in PBS and analyzed using FACS flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) and Cell Quest software.

2.4 Breast Cancer Stem Cell Culture

Breast cancer stem cells (named VNBRCA) were isolated following to the previous published reseach [30]. VNBRCA cell line was cultured in DMEMF12 (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated FBS, 2 mML-Glutamine and 100 U/ml of penicillin at 37ºC, 5% CO_2. Fresh medium was additionally provided at a 3-day interval to provide nutrients. VNBCRA was stained with APC-labeled anti-CD44 and FITC anti-CD14 antibodies. Flow cytometry protocol was used for analyzing the surface markers of VNBRCA as described in the foregoing.

2.5 Generation of Hybrid Cells by Electrofusion

Electrofusion is the process of combining two cell types using an electrical field. The short, intense pulses created increase the permeability of the membranes as well as the membrane flow. The resulting local perforation of the cell membrane allows cell fusion, forming a new cell type with the properties of the two partners.

Cells from cultures were detached from plastic culture flasks using 0.05% trypsin /0.02% EDTA solution washed twice in PBS, spun at 170×g for 7 min and delivered into a pre-cooled low conductive medium, with a pH of 7.34 to 7.40, by repeating cell suspension with three centrifugation steps (170 × g spin for 7 min, 18ºC). Cell number and viability were counted before and after transfer steps. DCs and tumor cells were mixed in a ratio of 1:1 to a total density of 5×10^5 cells/ml in a 250 µl Helix fusion chamber. Electrofusion was then performed by a Multiporator machine (Eppendof, Hamburg, Germany) with the following parameters: Alignment: 9 V, 30 s; Pulse: 95 V, 30 µs, 2 pulses; post-alignment: 8 V, 30 s.

2.6 Characterization of Fusion Cells

FCs were visualized by fluorescence microscope and the marker surface was analyzed by flow cytometry. To visualize fusion hybrids, before fusion, VNBRCA membrane was stained with VybrantDil (0.5 µg/ml) in DMEMF12 at 37ºC for 24 h. Vybrant Dil is a lipophilic membrane stain that diffuses laterally to stain the entire cell. It is weakly fluorescent until it is incorporated into membranes giving a strong red fluorescence light at 565 nm.

DCs were cultured in RPMI and incubated with Hoechst 33342 at 37ºC for 12 h to stain the nucleus. The Hoechst is part of a family of fluorescent stains for labeling DNA. Because these fluorescent stains label DNA, they are also commonly used to visualize the nuclei. Hoechst 33342 emit blue/cyan fluorescence light around an emission maximum at 461 nm. After fusion, the cell suspensions were then confirmed by inverted flourescence microscopy.

For marker analysis of FCs and evaluating the fusion efficiency, the cell suspensions after fusions were stained with anti-CD80-FITC and CD44-PE antibodies. After washing 3 times with PBS, cells were analyzed by flow cytometry.

2.7 T-cells Proliferation Assay

T-cell proliferation aimed to evaluate the number and function of T-cell proliferation in the culture. MTT was added to the culture to measure the proliferation of T-cell in response to antigens. In this study, DCs and FCs (stimulator cells) were incubated with CD8+ T lymphocytes (responder cells) at different ratios in 96-well round bottom culture plates. T-cells were also incubated with 5 µg/ml phytohaemagglutinine (PHA) as a positive control. PHA has effects on cell metabolism and is used as a mitogen to trigger cell division in T-lymphocytes.

The plates were incubated 24 h in a humidified atmosphere under 5% CO_2 at 37ºC. Cells were co-incubated at responder: stimulator (R:S) ratios of 1:1, 2:1, 4:1, 8:1, and 16:1. Proliferation of lymphocytes was determined by adding the MTT within the last 1 h of incubation. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] was reduced to form purple formazan in living cells. DMSO was added to dissolve the purple formazan crystal. The absorbance of this colored solution was then

measured at a 500 to 600 nm wavelength by a spectrophotometer DTX 880 (Beckman Coulter, America).

3. RESULTS

3.1 Characterization of Dendritic Cells (DCs)

Before and after monocyte induction for DC specialization, surface marker of DCs was analyzed by flow cytometry to determine whether DCs are successfully differentiated or not. Characteristics of MHC class II like CD40, CD80 and CD86 are notable for DCs.

The results show that, before induction, the cells are negative with maker MHC class II like HLA-DR, HLA-DQ, CD40, CD80 and CD86 (Fig. 1). After induction, most of the cells were positive with CD40 (83.19%), CD80 (72.70%), CD86 (74.79%), HLA-DR (81.54%) and HLA-DQ (86.54%) (Fig. 1), though the efficacy given by Cell Quest software was nearly 100%. This indicated that we were able to successfully differentiate umbilical cord blood-derived monocytes into DCs.

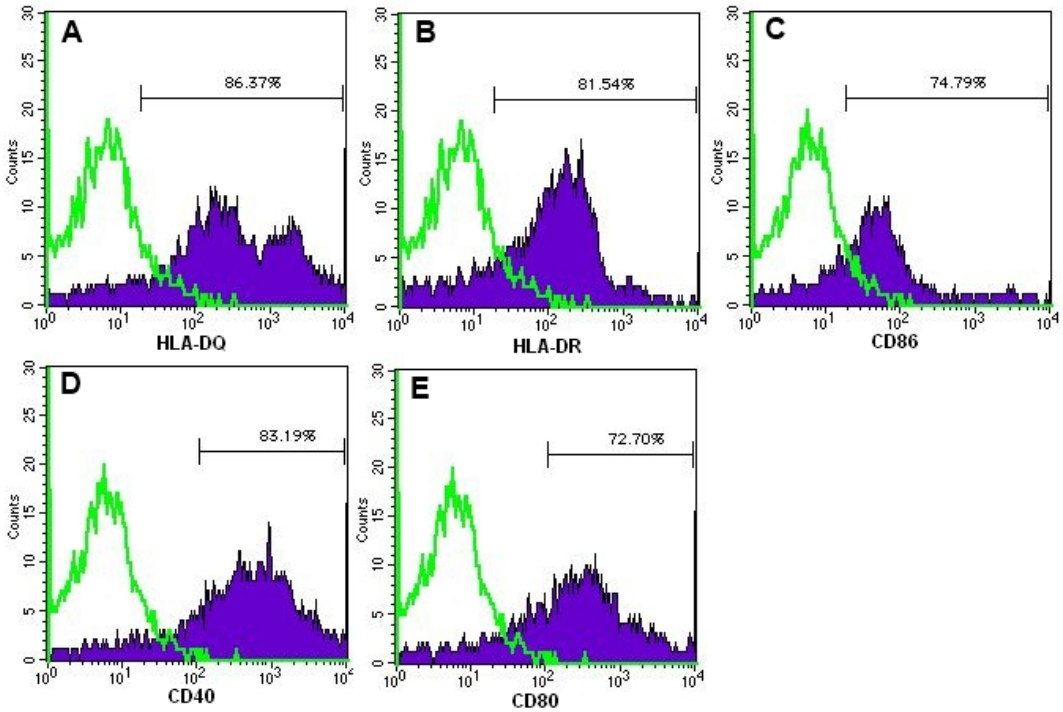

Fig. 1. Phenotypic characterization of DCs after induction by flow cytometry.
After inducing, monocytes expressed HLA-DQ (A), HLA-DR (B), CD86 (C), CD40 (D) and CD80 (E).

Moreover, monocytes are spherical in shape, after collection from the blood. After induction with GM-CSF and IL-4, monocytes were induced into immature DCs, spread out at the bottom of the flask and expressed in lozenge shape [22,38].

3.2 Characterization of Vietnamese Breast Cancer Stem Cell (VNBRCA)

Before the fusion experiment, as well as DCs, VNBRCA cell line was characterized with the purpose of following up easily the phenotype of FCs after the fusion experiment. VNBRCAs were stained with anti-CD24 and anti-CD44 antibodies, and the marker surface was then analyzed by flow cytometry. The result shows that VNBRCA expresses CD44+ and CD24- (Fig. 2). This finding is in agreement with the result of previous studies [29,30,39]. In these studies our group proved that VNBRCA with CD44+ and CD24- phenotypes exhibiting the breast cancer stem cell characteristics such as anti-tumor drug resistance, strong tumorigenicity in NOD/SCID mice…[29,39]

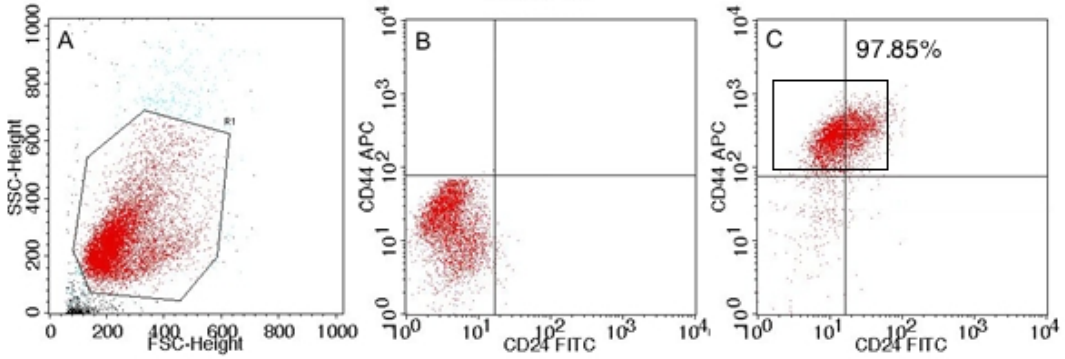

Fig. 2. Phenotypic characterization of VNBRCA by flow cytometry.
VNBRCA cells were stained with anti-CD44 and anti-CD24 antibodies. (A) Cells were gated in SSC and FSC histogram, (B) Labeled cells were detected by FL1 channel (for CD24-FITC) and FL4 channel (for CD44-APC) with isotype control stained cells as negative control (B).

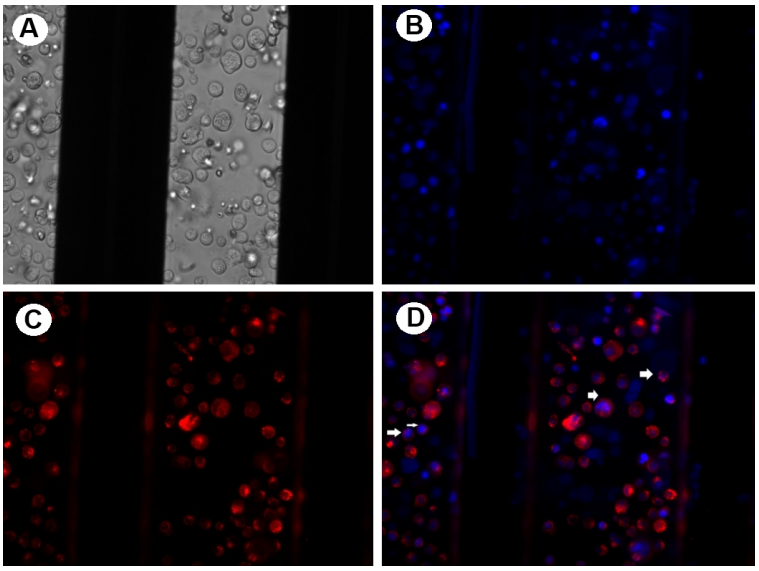

Fig. 3. FCs confirmed by fluorescence microscope.
VNBRCA membranes are stained with Vybrant-Dil, and DC nuclei are stained with Hoechst 33342. (A) Mixture of cells after fusion under light field, (B) Immunofluorescence of Hoechst-labeled DCs, (C) Immunofluorescence of Vybrant-Dil-labeled tumor cells, (D) Colocalization of both fluorescent image (B) and (C).

3.3 Characterization of FCs

Based on the phenotypes of the two partners' cell lines which are determined in previous experiments, FCs are easily recognized by analyzing dual phenotypes expression. The mix of cells after fusion was stained with anti-CD44 which is specific for VNBRCA, and anti-CD80 which is specific for DCs. The sample was then analyzed by flow cytometry, and it was expected that the FCs would express both maker surfaces CD44 and CD80. The result shows that 20% of cells express dual CD44 and CD80. This also indicated that the efficacy of FCs is 20%. Another method that can visualize fusion cells is the use of fluorescence microscope with specific stains. Before electrofusion, VNBCRA1's membranes were stained with Vybrant Dil Solution which expresses red colour at 565 nm. DC's nuclei were stained with Hoechst 33342 which expresses blue colour at 461 nm. After staining, the fusion experiments were performed. Cell mixture after fusion was then analyzed by fluorescence microscopy. The result shows that at appropriate wavelengths, DCs express blue color (Fig. 3B) and VNBRCA1s express red color (Fig. 3C). The FCs expressed both red and blue color (Fig. 3D). This result indicates again that we are successful in the generation of FCs from dendritic and breast cancer stem cells.

3.4 T-cell Proliferation

To determine whether or not FCs are effective in stimulating T-cells, FCs were co-cultured with T-cells isolated from fetal cord blood. To establish a control, T-cells were also co-cultured with DCs. T-cells were stimulated with PHA as a positive control. T-cell proliferation was measured by MTT assay. The result shows that stimulation of T-cells by FCs is the same as the positive control. T-cells stimulation by DCs is lower than that by FCs (Fig. 4). This indicated that FCs have the ability to present the antigen of breast cancer cell to stimulate T-cells proliferation.

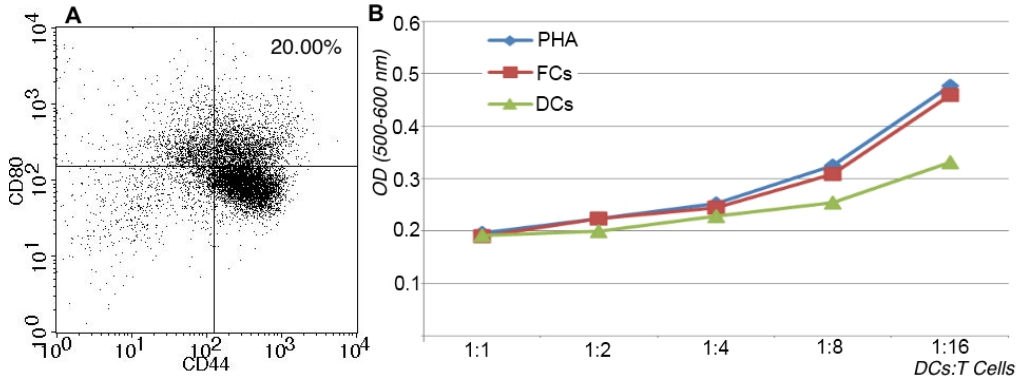

Fig. 4. (A) **Efficacy of FCs determined by flow cytometry**.
Cell mixture after electrofusion stained with anti-CD80-FITC and CD44-PE and analyzed by flow cytometry. (B) ***Stimulation of T-cells by FCs****. T-cells was co-cultured with DCs, FCs and PHA with the increasing ratios of T-cells.*

4. DISCUSSION

One of the reasons which led to the development of immune tolerance towards tumor cells, is that tumor antigens were presented to the immune system without the presence of

essential co-stimulatory signals necessary for the priming of T-cell responses [27,36]. Therefore, T-cells with the capability of recognizing these antigens cannot be activated. One of the major areas of cancer immunotherapy has been the attempt to enhance tumor-induced energy through presentation of an antigen in the context of appropriate secondary signals.

DCs are considered to be the professional antigen-presenting cells. DCs capture and process antigens into peptides for loading onto MHC class I and II, and present them to T-cells and B-cells. In DC/tumor fusion approach, multiple tumor antigens, including some yet unidentified, can be presented simultaneously in appropriate MHC context, and thus immunity with multiple tumor antigens should be more effective than with a single antigen. In this study, we provided some important features for the clinical development of DC-breast cancer cell fusion vaccines as follows: (1) a potential source for generation of DCs used in cancer vaccine was suggested, (2) it was shown that hybrids of VNBCRA and DCs can be generated by electrofusion, (3) fusion cells express tumor-associated antigens and are able to stimulate T-lymphocyte proliferation.

In most of the studies, DCs were generated from the bone marrow or monocytes in the peripheral blood [20,21,25,27,36]. We were successful in using blood from umbilical cord which is not only the potential rich source of monocytes, but also utilizes medical-waste. In our study, the rate of observed FCs generated by electrical stimulation was 20%. This rate was relatively the same as previous report using PEG [16], but lower than other studies using electrofusion. In general, the fusion rate has been reported to be 12 to 60% [23,33, 41]. Different conditions of electrical and non electrical parameters influence the fusion efficacy. The alignment of cells in the cuvette prior cell fusion by means of dipoles, generated in an alternating current field, led to close cells contact is a requirement of cell fusion. Indeed, in electrofusion, the most critical parameters affecting inversely hybrid cell yield are the strength and duration of electrical pulse. So, the strength and duration of no good electric can cause the decrease of fusion efficacy. Another very important agent is the electrofusion buffer. With the hypotonic buffer, the membranes and cytoskeletons are temporarily loosened, thus cell fusion in the electric field takes place much more efficiently. Hence, the nonoptimal fusion buffer can also cause decrease of fusion efficacy.

The result shows that the hybrid cells still carry the antigen provided by cancer cells and the co-stimulatory molecules provided by DCs, which are needed to induce activation and differentiation of naive cytotoxic T-lymphocytes. This could be the advantage of dendritic hybrid cell therapy compared to the conventional use of antigen presenting cells. Indeed, we demonstrated that FCs have the ability to present the antigen of breast cancer stem cell needed for T-cells stimulation. In this study, the proliferation of T-cells occurred after 1 day, while other studies measured the proliferation after 5 to 7 days of incubation [16,36]. However, in another study [15], FCs were cultured for 4 to 5 days before analyzing the marker surface and incubation with T-cells. In contrast, in this study, the FCs are analyzed just after fusion for 4 h and incubated immediately with T-cells. This suggested that the FCs that we generated have strong ability to express TAAs. However, there is still an open question regarding the functional properties of hybrid cells. The cell line used in the research is cancer stem cell line, which has the possibility of immortalization. Therefore, in further researches, the loss of immortalization should be ensured to avoid tumor induction before proceeding with any clinical application.

5. CONCLUSION

In summary, the aim of this work was to characterize fusion cells generated by DC and Vietnamese breast cancer stem cells. We demonstrated that imaging means are extremely reliable and necessary to complement flow cytometry for the characterization, and also showed that FCs are functional to activate T-cells effectively while DCs are not. Conservation of both phenotype of VNBRCA and DC indicates the potential of fused cells as a breast tumor vaccine based on dendritic cells. However, this study also existed some limitations. Especially, this study had not evaluated effects of FCs that DCs fused with other subset of cancer cells on T-cells activation. Moreover, VNBRCA population is heterogeneous population; hence, further experiments have to be carried out to evaluate more clearly the functionality of FCs from other subtypes of VNBRCA.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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