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ORIGINAL RESEARCH

Overexpress of CD47 does not alter the stemness of MCF-7 breast cancer cells

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Abstract— Background: CD47 is a transmembrane glycoprotein expressed on all cells in the body and particularly overexpressed on cancer cells and cancer stem cells of both hematologic and solid malignancies. In the immune system, CD47 acts as a "don't eat me" signal, inhibiting phagocytosis by macrophages by interaction with signal regulatory protein α (SIRPα). In cancer, CD47 promotes tumor invasion and metastasis. This study aimed to evaluate the stemness of breast cancer cells when CD47 is overexpressed. **Methods**: MCF-7 breast cancer cells were transfected with plasmid pcDNA3.4-CD47 containing the CD47 gene. The stemness of the transduced MCF7 cell population was evaluated by expression of CD44 and CD24 markers, anti-tumor drug resistance and mammosphere formation. **Results**: Transfection of plasmid pcDNA3.4-CD47 significantly increased the expression of CD47 in MCF-7 cells. The overexpression of CD47 in transfected MCF-7 cells led to a significant increase in the $CD44^+CD24^-$ population, but did not increase doxorubicin resistance of the cells or their capacity to form mammospheres. **Conclusion**: CD47 overexpression enhances the $CD44^+CD24^-$ phenotype of breast cancer cells as observed by an increase in the $CD44^+CD24^-$ expressing population. However, these changes are insufficient to increase the stemness of breast cancer cells.

Keywords: CD47, breast Cancer, breast cancer stem cells, MCF-7

INTRODUCTION

According to World Health Organization (WHO), breast cancer is the most common cancer in women both in the developed and developing nations with nearly 1.7 million cases diagnosed in 2012 (Ferlay et al., 2015). The main goal of cancer treatment is to cure or prolong the lifetime of patients and to ensure the best quality of life after treatments. By using a model in which human breast cancer cells were grown in immunocompromised mice, Al-Hajj *at el*. (2003) had found that only a minority of breast cancer cells had the ability to form new tumor (Al-Hajj et al., 2003). Recently, much evidence was provided that leukemia and solid tumors maintenance and growth are sustained by a small proportion of cells exhibiting stem cell properties (Nicolis, 2007; Reya et al., 2001; Visvader and Lindeman, 2008) which named tumor-initiating cells (TICs) or cancer stem cells (CSCs) (Clarke et al., 2006). CSCs are resistant to many conventional cancer therapies, chemotherapy and radiotherapyso they can survive after treatments in spite of shrinkage of the tumor which results to tumor relapse. They are difficult to kill because they overexpress drug efflux pumps and have an increased capacity to active anti-apoptotic and pro-survival pathways as well as DNA repair (Flemming, 2015). Actually, to ensure completely cure cancer, many new cancer therapies now are focused on the targeting CSCs. Recently, some studies showed that cancer stem cells could escape the imunne system by high expres-

sion of CD47 on their cell surface (Cioffi et al., 2015; Zhang et al., 2015).

Indeed, CD47 is expressed on almost all human cells as evaluated by mRNA expression and immunohistochemical staining. CD47 was first identified as an antigen expressed on ovarian carcinoma cells (Mawby et al., 1994). CD47 is overexpressed in many solid tumors as well as hematologic malignancies (Chao et al., 2010a; Chao et al., 2010b; Chao et al., 2012; Jaiswal et al., 2009; Majeti et al., 2009). CD47 is involved in the regulation of various activities of different immune cell types and can induce direct cancer cell death when it is crosslinked (Oldenborg, 2004). In addition, CD47 plays an important role in angiogenesis, proliferation and cell adhesion (Brown and Frazier, 2001; Reinhold et al., 1995). The interaction between CD47 on T-cells and its receptor SIRP-α on dendritic cells (DCs) is part of a new regulatory pathway that may be involved in the maintenance of homeostasis by preventing the escalation of the inflammatory immune response (Latour et al., 2001). Anti-CD47 antibody treatment not only enables macrophage-mediated phagocytosis of cancer cells but also can initiate an antitumor cytotoxic T-cell immune response (Tseng et al., 2013). CD47 expression is elevated in human non-Hodgkin lymphoma (NHL); blocking anti-CD47 antibodies preferentially enabled phagocytosis of NHL cells and synergized with rituximab (Chao et al., 2010a).

Therefore, we hypothesized that CD47 holds the important role in cancer stem cell formation. Therefore, this study aimed to over-express the CD47 in breast cancer cells and avaluate their stemness as breast cancer stem cells.

MATERIAL AND METHODS

Cancer cell lines

Breast cancer cell lines (MCF-7 and MDA-M-231) were obtained from ATCC and VNBRC1; S6 cell line was established by our laboratory (Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, Ho Chi Minh city, Vietnam). All cell lines were thawed from nitrogen and cultured for several passages to confluency prior to use in experiments.

Cell culture

Cells were cultured in DMEM/F12 complete (i.e. DMEM/F12 with 10% fetal bovine serum and 1% antibiotic-mycotic; all reagents purchased from Sigma-Aldrich, St Louis, MO) in a 37^{o}C, 5% CO_2 incubator.

Cloning pcDNA3.4-CD47 plasmid

CD47 gene was isolated from cDNA of mRNA from S6 cells by PCR reaction with full-length primers under thermal cycle: denaturation 95^{0}C / 5 minutes - 35 cycles (95^{0}C / 20 seconds - 59^{0}C / 30 seconds - 72^{0}C / 1 min) - lasted 72^{0}C / 10 minutes. Then, fresh PCR products were ligated with linear pcDNA3.4 vector (Life Technologies, USA) by TA cloning reaction and Topoisomerase enzyme which available in vector pcDNA3.4. The ingredient of ligation reaction followed **Table 1**.

Table 1. Cloning reaction of pcDNA3.4-CD47 plasmid

Reagent	Volume (μl)
PCR product of CD47 gene	2
Salt solution (NaCl, $MgCl_2$)	1
pcDNA3.4 TOPO vector	1
Sterile water	to 6μl

Transient transfection of DNA plasmid

DNA plasmids were transiently transfected by electroporation on a Multiporator® Electroporation system (Eppendorf, Hamburg, Germany) following the manufacturer's instructions. In brief, MCF-7 cells were trypsinized, adjusted to a density of 0.5-1 x10^6 cells/mL in 400 μL Hypoosmolar Solution (Eppendorf), and incubated with 20 μg/mL DNA plasmid. The electroporation was performed at 300 V in 40 μs. After electroporation, transfected cells were cultured in DMEM/F12 containing 10% FBS in a 37^{o}C, 5% CO_2 incubator. After 48 h of incubation, cells were analyzed for expression of CD47 and stemness-related genes, formation of spheres, and anti-tumor drug resistance.

Real-time RT-PCR

Approximately 5x10^6 cells were obtained for total RNA isolated using EasyBlue total RNA extraction kit (iNtRON, Korea) according to the manufacturer's instructions. The concentration of total RNA were measured with a Biophotometer (Eppendorf). Real-time one step RT-PCR was carried out using a 2x qPCR SyGreen 1-Step Mix Lo-ROX (PCR BioSystems, London, UK). Relative quantification was estimated by the Livak method, $2^{-\Delta\Delta Ct}$.

Table 2. Primer sequences used to analyzed stemness of MCF-7 cells after transfected pcDNA3.4-CD47 plasmid

Genes	Primer sequences (5'-3')	Size
GAPDH	GAGTCAACGGATTTGGTCGT	238 bp
	TTGATTTTGGAGGGATCTCG	
CD47	GGCAATGACGAAGGAGGTTA	217 bp
	ATCCGGTGGTATGGATGAGA	
CD44	AGTGAAAGGAGCAGCACTTCAG	151 bp
	TCTCAGTTGCTGTAGCACTAG	
CD24	ATGGGCAGAGCAATGGTGGC	156 bp
	ATTAGTTGGATTTGGGGCCAAC	
SOX 2	ACGGCAGCTACAGCATGATGC	150 bp
	TCATGTAGGTCTGCGAGCTGG	
NANOG	TGTGGGCCTGAAGAAAACTATCC	150 bp
	AGTGGGTTGTTTGCCTTTGGG	
OCT4	AGGGCTCTTTGTCCACT	256 bp
	ACTCTCCCCAGCTTGCTT	
cMYC	GTCGTTTCCGCAACAAGTCCTC	112 bp
	AATGAAAAGGCCCCCAAGGTA	

Flow Cytometry

CD47 expression and the ratio of breast cancer cell populations were analyzed based on the percentage of cells expressing these markers: CD47, CD44 and CD24. CD44 is a marker of cancer stem cells (Baumann and Krause, 2010) and CD24 is considered to be a marker for breast cancer cells (Schabath et al., 2006). Cultured cells were trypsinized into single cells and diluted in 100 μL FACSflow solution at a density of 10^6 cells/mL. Then cells were stained with CD47-FITC (Sigma-Aldrich), CD24-FITC (BD Biosciences, San Jose, CA) and CD44-APC antibodies (BD Biosciences). Cells were analyzed by flow cytometry on a FACSCalibur machine (BD Biosciences) using CellQuest Pro software (BD Biosciences).

Immunocytochemistry

Cells were cultured in 48-well plates at a cell density of 10^5 cells/well for 24 h before use in experiments. Cells were fixed with 4% paraformaldehyde and washed three times with cold PBS. Fixed cells were incubated with anti-CD47 FITC-conjugated Ab (Sigma-Aldrich, Louis St, MO), anti-CD24 FITC-conjugated Ab (BD Biosciences), and/or anti-CD44 PE-conjugated Ab (BD Biosciences). Cell nuclei were stained with Hoeschst 33442 (Sigma-Aldrich) and images were captured by a Carl Zeiss AxioLab A1 microscope (Carl Zeiss, Germany).

Sphere formation assay

Cells were plated at 10^3 cells/mL in serum-free DMEM/F12 medium supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 5 ng/mL insulin (Santa Cruz Biotechnology, Santa Cruz, CA), and 0.4% bovine serum albumin (BSA; Sigma-Aldrich). Cells cultured under these conditions were capable of forming spherical clusters of cells ("spheres" or "mammospheres"). The number of spheres in each well was evaluated after 7 d of culture.

Anti-tumor drug assay

Non-transfected MCF-7 and CD47-transfected MCF-7 cells were plated at a density of 10^4 cells/well in E-plate 96 (ACEA Biosciences, San Diego) in DMEM/F12 supplemented with 10% FBS. After 24 h, cells were treated with 0, 1, 3, 6, or 9 μg/mL doxorubicin (Sigma-Aldrich) for 48 h. The cells were evaluated by an xCELLigence RTCA SP system for proliferation and drug resistance (IC_{50} index).

Statistical analysis

All experiments were performed in triplicate. Data were analyzed by Grapad Prism 6.0 software. The significant differences between mean values were assessed by the t-test and ANOVA, with P-value < 0.05 was considered to be significant.

RESULTS

Morphology and CD47 gene expression in four breast cancer cell lines (MCF-7, MDA-MB-231, VNBRC1 and S6)

To determine CD47 expression in cancer cell lines, cells were cultured for real-time RT-PCR analysis using specific primers for CD47. When cultured as an adherent monolayer, MCF-7 cells exhibited an

epithelial-like morphology (i.e. bean shape) while MDA-MB-231 cells exhibited a mesenchymal-like, spindle shape. VNBRC1 and S6 cells had an epithelial-like morphology. When observed under microscopy, MCF-7 cells were smaller in size than other three cancer cell lines (**Fig. 1A**). CD47 gene expression was evaluated by real-time RT-PCR in three independent experiments. The results showed that when compared to CD47 mRNA expression by peripheral blood cells, the CD47 expression level by VNBRC1 cells was similar (average level), while expression by S6 cells was significantly higher (13556±67 fold) and by MDA-MB-231 cells was significantly lower (0.017±0.09 fold) (**Fig. 1B**).

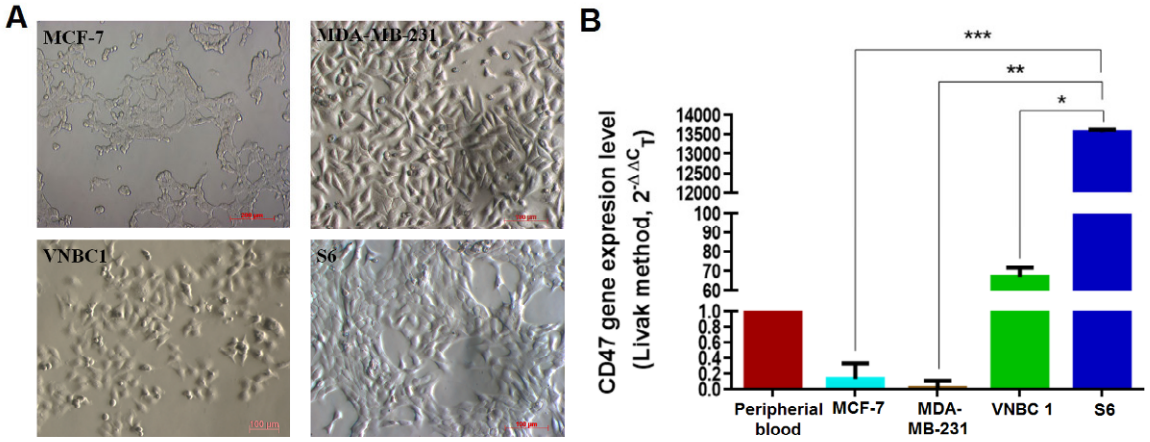

Figure 1. Morphology of various cancer cell lines in vitro and their CD47 mRNA expression. (A) Morphology of four breast cancer cell lines (MCF-7, MDA-MB-231, VNBRC1 and S6) in vitro. (B) CD47 mRNA expression level of the four breast cancer cell lines relative to peripheral blood cells compared to its expression in peripheral blood cells (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Expression of CD47, CD44, and CD24 cell populations in the four breast cancer cell lines

After trypsinization, expression of CD47, CD44 and CD24 markers was assessed on the four breast cancer cell lines by flow cytometry and immunocytochemistry. The results of flow cytometry showed that there are significant differences in CD47 expression by S6 and VNBRC1 cells, VNBRC1 and MDA-MB-231 cells, MDA-MB-231 and MCF-7 cells, and MCF-7 and S6 cells (97.38±0.35% vs. 93.6±0.42%, 93.6±0.42% vs. 87.91±1.16%, 87.91±1.16% vs. 74.86±1.34%, and 74.86±1.34% vs. 97.38±0.35%, respectively) ($p < 0.05$) (**Fig 2.A**). Moreover, there were significant differences by flow in the percentage of the $CD44^{+}CD24^{-}$ cell population for MDA-MB-231 vs. MCF-7, MDA-MB-231 vs VNBRC1, MDA-MB-231 vs S6, and S6 vs MCF-7 (18.62±0.64% vs. 0%, 18.62±0.64% vs. 1.64±0.1%, 18.62±0.64% vs. 1.92±0.16%, and 1.92±0.16% vs 0%, respectively ($p < 0.05$) (**Fig 2.B**).

FACS results confirmed CD47 protein expression by the four cancer cell lines (**Fig. 2C**) as well as their co-expression of CD44 and CD24. Further analysis showed that MCF-7 cells and S6 were predominantly CD44 positive and CD24 negative; the percent of cells that were CD44+CD24- in the MCF-7 cell line and the S6 cell line were 69.18±3.24 % and 60.15±0.97%, respectively. The percent of MDA-MB-231 cells with the CD44+CD24- phenotype was 47.52±0.48%, while the percent for VNBRC1 cells was 61.72±1.91%.

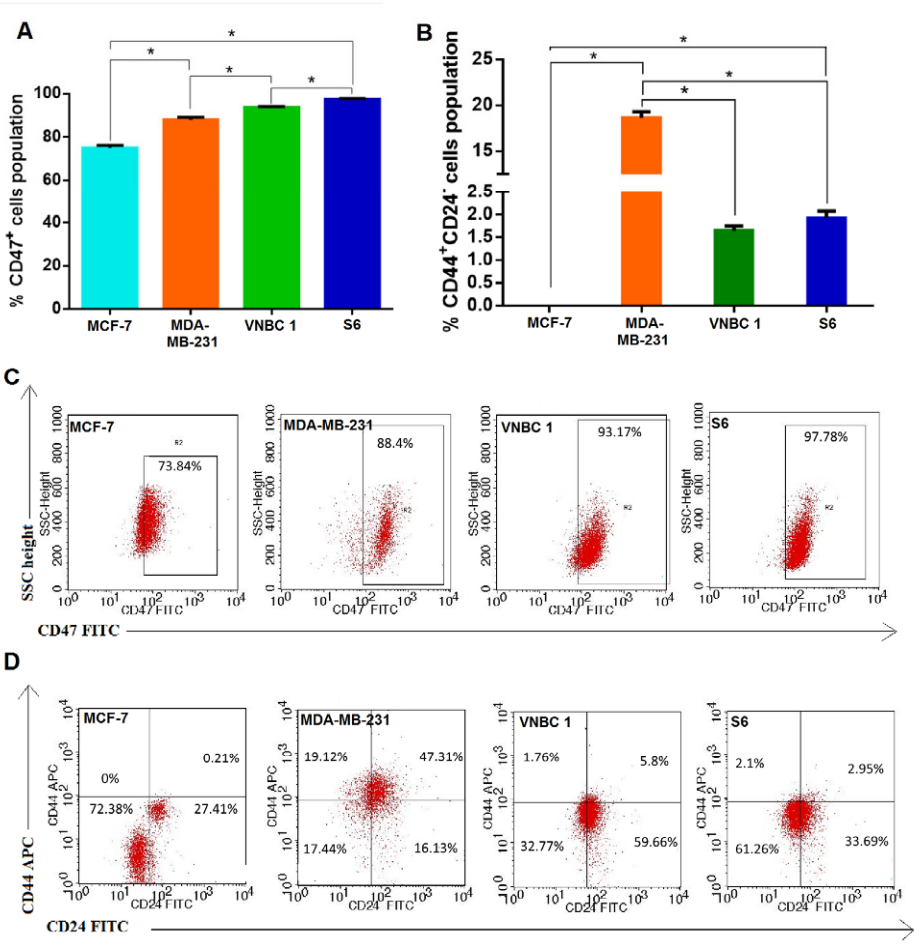

Figure 2. FACS analysis of CD47, CD44 and CD24 expression in four human breast cancer cell lines (MCF7, MDA-MB-231, VNBRC1, S6). (A) Chart showing the percentage of $CD47^+$ cell population; (B) Chart showing the percentage of the $CD44^+CD24^-$ population (with *: $p < 0.05$); (C) FACS analysis for single staining with human anti-CD47 FITC-conjugated Ab; (D) FACS analysis for double staining with anti-CD44 APC-conjugated and anti-CD24 FITC-conjugated Abs. Each cancer cell line shows differential protein expression of CD47, CD44 and CD24.

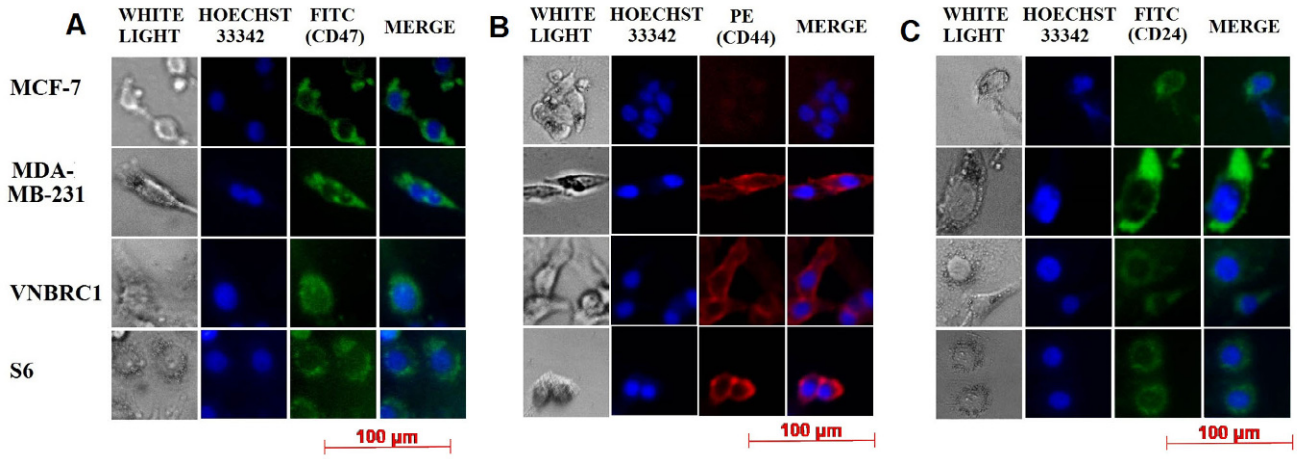

Figure 3. Expression of CD47, CD24 and CD44 on four breast cancer cell lines as identified by immunocytochemistry. CD47 expressed almost in all cell lines (A), while CD44 expressed on MDA-MB231, VNBRC1, S6 but not in MCF7 (B), and CD24 weakly expressed in MCF-7, VNBRC1, S6 but highly expressed in MDA-MB231.

The results of immunocytochemistry showed that all cancer cell lines express CD47 (**Fig 3A**). All four cancer cell lines evaluated in this study were positive for CD24 with MDA-MB-231 showing the greatest expression. The MCF-7 cell line was negative for the CD44 marker and only weakly positive for CD24; these results are similar to flow cytometry results for MCF-7 cells (0% $CD44^{+}CD24^{-}$ cell population). The MDA-MB-231 cell line is positive for all three markers with a strong FITC signal for the CD24 marker; notably, VNBRC1 and S6 cell lines are also positive for all three markers, with an especially strong signal for CD47 and CD44.

Expression of CD47 in CD47-transfected MCF-7 cells

Following pcDNA3.4-CD47 plasmid transfection, we performed protein quantification by flow cytometry. The results showed that the percent of CD47 positive cells increased significantly from 74.86±1.34% (non-transfected) to 96.43 ± 0.53% (transfected) (n=3 independent trials, $p < 0.05$) (**Fig 4.A,B**). Flow cytometry results for CD44 and CD24 expression were shown in **Table 1**. There were also significant differences in CD44 and CD24 expression by MCF-7 cells versus by transfected MCF-7 cells (called MCF-7-TF) (**Fig. 4C,D**). Taken together, our results suggest that transfection efficiency can be enhanced via CD47, CD44 and CD24 markers on cell surfaces of MCF-7 cells.

Table 3. CD44+CD24- phenotype in MCF-7 cells and CD47-transfected MCF-7 cells (MCF-7-TF).

	$CD44^{+}CD24^{-}$ (*)	$CD44^{+}CD24^{+}$ (*)	$CD44^{-}CD24^{-}$ (*)	$CD44^{-}CD24^{+}$(*)
MCF-7	0	0.22 ± 0.05	69.18 ± 3.24	30.59 ± 3.19
MCF-7-TF	2.41±0.26	0.46 ± 0.12	95.79 ± 1.1	1.34 ± 0.73

** $p < 0.05$*

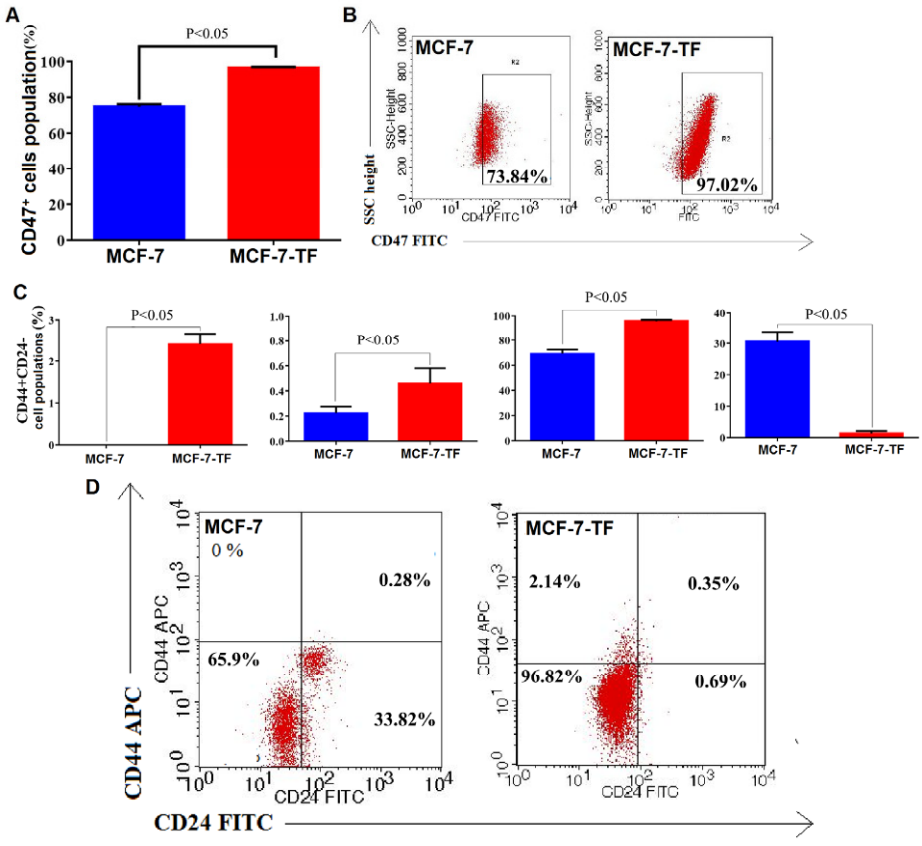

Figure 4. Gene expression and FACS analysis for single stain CD47 and double stain CD44 and CD24 in MCF7 cells before and after transfection. MCF7 significantly expressed CD47 after transfection with plasmid in transcriptional level (A) and translational level (B). The $CD44^{+}CD24^{-}$ cell population in MCF7 cells also increased after transfected with CD47 plasmid (C, D). MCF7: MCF7 cells before CD47 gene transfection, MCF7-TF: MCF7 cells after CD47 gene transfection for 48 h

Gene expression of several stemness-related genes

To investigate the increase of CD47 expression, total RNA was extracted to evaluate mRNA gene expression. In this study, we analyzed several genes related to CD47 and stemness in CD47-transfected MCF-7 cells (MCF-7-TF). We used normal MCF-7 cells as a negative control. This experiment was performed for 7 genes which profile is associated to stemness, and with GAPDH as an internal control. Real-time RT-PCR were performed on 3 independent experiments and the relative quantitative results were analyzed by Livak method, $2^{-\Delta\Delta C_T}$.

As presented in **Fig. 5A**, the expression of CD47 by MCF-7-TF (transfected) cells is higher than that for MCF-7 cells alone. The expression of OCT4, cMYC, SOX2, and NANOG by MCF-7-TF cells are higher than that for non-transfected MCF-7 cells. There are significant differences in CD47 expression between MCF-7 and MCF-7-TF cells (22.95±5.53 vs. 1), respectively. Moreover, there are similarities in the "stemness" gene expression of OCT4, cMYC, SOX2, NANOG, CD44, and CD24 in MCF-7 and MCF-7-TF cells (1.37±0.44 vs. 1; 1.33±1.09 vs. 1; 1.25±1.12 vs. 1; 2.14±1.74 vs 1, 0.85±0.51 vs. 1, and 1.17±0.13 vs 1, respectively) ($p<0.05$). These results demonstrate that upregulation of CD47 expression is not enough to alter the expression of several stemness genes in MCF-7 cells, compared to pcDNA3.4-CD47 plasmid transfected MCF-7 cells.

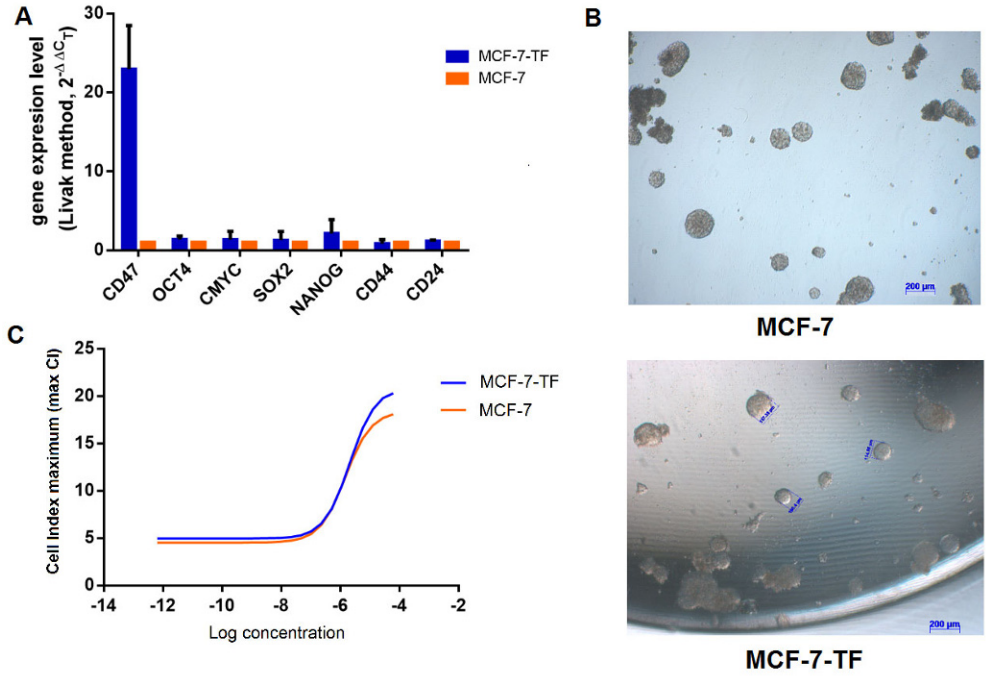

Figure 5. Results of gene expression, sphere formation and anti-tumor drug assay in CD47-transfected MCF-7 cells.

Sphere formation in serum-free medium

MCF-7 cells could not adhere onto the flask surface when cultured in serum-free medium nor could they form spheres (**Fig. 5B**). There were similarities in the number of spheres in the MCF-7-TF group and MCF-7 group (95±12.6 and 98.67±26.4 spheres, respectively); however, there was no statistically significant difference ($p=0.9062$).

Drug resistance of MCF-7 after transfection with pcDNA3.4-CD47 plasmid

Doxorubicin had a suppressive effect on proliferation of breast cancer cells. In the absence of doxorubicin, proliferation rates were similar after 48 h in culture for non-transfected and CD47-transfected MCF-7 cell groups. There was some similarity in the IC_{50} index of non-transfected MCF-7 and MCF-7-TF (1.506 μg/mL and 2.1 μg/mL) (**Fig. 5C**).

DISCUSSION

From assessment of cell morphology, the results showed that three breast cancer cell lines (MCF-7, VNBRC1 and S6) are similar in that they have an epithelial-like shape. Conversely, the MDA-MB-231 cell line has a mesenchymal-like shape (spindle shape). The ratio of the $CD44^+CD24^-$ population to the entire cell population for the VNBRC1 and S6 cell lines are similar to what has been described; some studies, too, have reported that less than 2% of the cell population have the $CD44^+CD24^-$ phenotype (Al-Hajj et al., 2003; Appalaraju Jaggupilli, 2012; Lapidot et al., 1994). CD47 expression in MCF-7 and MDA-MB-231 cells are similar to the published results of Manna et al. (2004); expression was monitored using an anti-CD47 antibody (BD Biosciences) (Manna and Frazier, 2004).

There are significant differences in CD47 expression and the $CD44^+CD24^-$ cell population of breast cancer cell lines. These differences may be caused by the origin and classification of the cancer cell lines. MCF-7 cells were derived from luminal epithelial cells while MDA-MB-231 cells were derived from basal epithelial cells so each cell line has a distinguishing gene profile (Holliday and Speirs, 2011; Kaur et al., 2015). CD47 mRNA and protein sequences are conserved among humans, mice and rats (Chang and Huang, 2004). In this study, we showed the relationship between CD47 expression and the ratio of $CD44^+CD24^-$ cells population in breast cancer cells. Taken together, the results demonstrated that S6 is a cell line with the highest CD47 expression; meanwhile, MCF-7showed the lowest CD47 expression among the four cell lines.

We used S6 cells for isolating the CD47 gene for subsequent cloning into the pcDNA3.4 vector to create a pcDNA3.4-CD47 plasmid. This plasmid was transfected into MCF-7 cells to induce upregulation of CD47 expression by MCF-7 cells. CD47 overexpression in MCF-7 cells was carried out by transfection of pcDNA3.4-CD47 plasmid into the cells. While electroporation is also a feasible procedure for gene delivery, DNA plasmid transfection was quite effective. In our study, the non-transfected MCF-7 cells did not express a $CD44^+CD24^-$ population, similar to the results published by Sheridan at el. (2006) (Sheridan et al., 2006). On the contrary, the transfected cells (MCF-7-TF) showed an increase in the $CD44^+CD24^-$ cell population. We also found that CD47 expression is related to the ratio of the $CD44^+CD24^-$ cell population; the changes in CD47 expression may cause changes in cell surface makers, thereby changing the phenotype of the cell population. However, these phenotypic changes could not modify cellular properties, such as drug resistance and mammosphere formation.

Gene expression of NANOG, OCT3/4, and SOX2 (stemness transcription factors) have been implicated in the progression of breast cancer (Apostolou et al., 2012; Apostolou et al., 2015). The oncogene cMYC is a transcription factor which regulates and initiates the immune regulator, CD47 (Casey et al., 2016); overexpression of CD47 may impact the regulation by cMYC. Mammosphere formation in culture was used to study stem cell properties. It was noted that expression of SOX2 (but not OCT4 or NANOG) was induced; in fact, overexpression of SOX2 increased mammosphere formation and activation of breast cancer stem cells (Leis et al., 2012).

According to particular properties of breast cancer stem cells, some breast cancer cells that are considered as breast cancer stem cells should exhibit stemness properties including both markers and biological activities. In our study, the cells either exhibited or promoted the $CD44^+CD24^-$ phenotype, strong anti-tumor drug resistance, mammosphere formation, and tumors in NOD/SCID mice (Jiao et al., 2016; Pham et al., 2011; Phan et al., 2016; Saadin and White, 2013). Therefore, although overexpression of CD47 caused formation of the $CD44^+CD24^-$ cell population, the cells did not promote the phenotypes of breast cancer stem cells. We propose that there are other signals besides CD47 which can alter breast cancer cells towards breast cancer stem cells.

CONCLUSION

The role of cancer stem cells in cancer recurrence and drug resistance is well-known. However, the relationship between cell surface markers and stemness properties of cancer stem cells is still unclear. This study showed that CD47 expression was related to formation of the $CD44^+CD24^-$ cell population in MCF7 breast cancer cells. However, overexpression of CD47 could not change the anti-tumor drug resistance as well as impede mammosphere formation of these cells. Therefore, CD47 gene is not enough to induce MCF7 breast cancer cells to become breast cancer stem cells.

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Competing Interests

The authors declare they have no competing interests.

References

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 100, 3983-3988.

Apostolou, P., Toloudi, M., Chatziioannou, M., Ioannou, E., and Papasotiriou, I. (2012). Cancer stem cells stemness transcription factors expression correlates with breast cancer disease stage. *Curr Stem Cell Res Ther* 7, 415-419.

Apostolou, P., Toloudi, M., and Papasotiriou, I. (2015). Identification of genes involved in breast cancer and breast cancer stem cells. *Breast Cancer (Dove Med Press)* 7, 183-191.

Appalaraju Jaggupilli, E.E. (2012). Significance of CD44 and CD24 as Cancer Stem Cell Markers: An Enduring Ambiguity. *Clinical and Developmental Immunology* 2012, 11.

Baumann, M., and Krause, M. (2010). CD44: a cancer stem cell-related biomarker with predictive potential for radiotherapy. *Clin Cancer Res* 16, 5091-5093.

Brown, E.J., and Frazier, W.A. (2001). Integrin-associated protein (CD47) and its ligands. *Trends in Cell Biology* 11, 130-135.

Casey, S.C., Tong, L., Li, Y., Do, R., Walz, S., Fitzgerald, K.N., Gouw, A.M., Baylot, V., Gutgemann, I., Eilers, M., *et al.* (2016). MYC regulates the antitumor immune response through CD47 and PD-L1. *Science* 352, 227-231.

Chang, W.T., and Huang, A.M. (2004). Alpha-Pal/NRF-1 regulates the promoter of the human integrin-associated protein/CD47 gene. *J Biol Chem* 279, 14542-14550.

Chao, M.P., Alizadeh, A.A., Tang, C., Myklebust, J.H., Varghese, B., Gill, S., Jan, M., Cha, A.C., Chan, C.K., Tan, B.T., *et al.* (2010a). Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* 142, 699-713.

Chao, M.P., Jaiswal, S., Weissman-Tsukamoto, R., Alizadeh, A.A., Gentles, A.J., Volkmer, J., Weiskopf, K., Willingham, S.B., Raveh, T., Park, C.Y., *et al.* (2010b). Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci Transl Med* 2, 63ra94.

Chao, M.P., Weissman, I.L., and Majeti, R. (2012). The CD47-SIRPalpha pathway in cancer immune evasion and potential therapeutic implications. *Curr Opin Immunol* 24, 225-232.

Cioffi, M., Trabulo, S., Hidalgo, M., Costello, E., Greenhalf, W., Erkan, M., Kleeff, J., Sainz, B., Jr., and Heeschen, C. (2015). Inhibition of CD47 Effectively Targets Pancreatic Cancer Stem Cells via Dual Mechanisms. *Clin Cancer Res* 21, 2325-2337.

Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H.M., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M. (2006). Cancer Stem Cells—Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer Research* 66, 9339-9344.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136, E359-386.

Flemming, A. (2015). Cancer stem cells: Targeting the root of cancer relapse. *Nat Rev Drug Discov* 14, 165.

Holliday, D.L., and Speirs, V. (2011). Choosing the right cell line for breast cancer research. *Breast Cancer Res* 13, 215.

Jaiswal, S., Jamieson, C.H., Pang, W.W., Park, C.Y., Chao, M.P., Majeti, R., Traver, D., van Rooijen, N., and Weissman, I.L. (2009). CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell* 138, 271-285.

Jiao, X., Rizvanov, A.A., Cristofanilli, M., Miftakhova, R.R., and Pestell, R.G. (2016). Breast Cancer Stem Cell Isolation. *Methods Mol Biol* 1406, 121-135.

Kaur, S., Singh, S., Elkahloun, A., Wu, W., Song, T., Arora, A., and Roberts, D. (2015). Role of CD47 in triple negative breast cancer. *The FASEB Journal* 29.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 367, 645-648.

Latour, S., Tanaka, H., Demeure, C., Mateo, V., Rubio, M., Brown, E.J., Maliszewski, C., Lindberg, F.P., Oldenborg, A., Ullrich, A., *et al.* (2001). Bidirectional negative regulation of human T and dendritic cells by CD47 and its cognate receptor signal-regulator protein-alpha: down-regulation of IL-12 responsiveness and inhibition of dendritic cell activation. *J Immunol* 167, 2547-2554.

Leis, O., Eguiara, A., Lopez-Arribillaga, E., Alberdi, M.J., Hernandez-Garcia, S., Elorriaga, K., Pandiella, A., Rezola, R., and Martin, A.G. (2012). Sox2 expression in breast tumours and activation in breast cancer stem cells. *Oncogene* 31, 1354-1365.

Majeti, R., Chao, M.P., Alizadeh, A.A., Pang, W.W., Jaiswal, S., Gibbs, K.D., Jr., van Rooijen, N., and Weissman, I.L. (2009). CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell* 138, 286-299.

Manna, P.P., and Frazier, W.A. (2004). CD47 mediates killing of breast tumor cells via Gi-dependent inhibition of protein kinase A. *Cancer Res* 64, 1026-1036.

Mawby, W.J., Holmes, C.H., Anstee, D.J., Spring, F.A., and Tanner, M.J. (1994). Isolation and characterization of CD47 glycoprotein: a multispanning membrane protein which is the same as integrin-associated protein (IAP) and the ovarian tumour marker OA3. *Biochem J* 304 (Pt 2), 525-530.

Nicolis, S.K. (2007). Cancer stem cells and "stemness" genes in neuro-oncology. *Neurobiology of Disease* 25, 217-229.

Oldenborg, P.A. (2004). Role of CD47 in erythroid cells and in autoimmunity. *Leuk Lymphoma* 45, 1319-1327.

Pham, P.V., Phan, N.L., Nguyen, N.T., Truong, N.H., Duong, T.T., Le, D.V., Truong, K.D., and Phan, N.K. (2011). Differentiation of breast cancer stem cells by knockdown of CD44: promising differentiation therapy. *J Transl Med* 9, 209.

Phan, N.L., Trinh, N.V., and Pham, P.V. (2016). Low concentrations of 5-aza-2'-deoxycytidine induce breast cancer stem cell differentiation by triggering tumor suppressor gene expression. *Onco Targets Ther* 9, 49-59.

Reinhold, M.I., Lindberg, F.P., Plas, D., Reynolds, S., Peters, M.G., and Brown, E.J. (1995). In vivo expression of alternatively spliced forms of integrin-associated protein (CD47). *J Cell Sci* 108 (Pt 11), 3419-3425.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* 414, 105-111.

Saadin, K., and White, I.M. (2013). Breast cancer stem cell enrichment and isolation by mammosphere culture and its potential diagnostic applications. *Expert Rev Mol Diagn* 13, 49-60.

Schabath, H., Runz, S., Joumaa, S., and Altevogt, P. (2006). CD24 affects CXCR4 function in pre-B lymphocytes and breast carcinoma cells. *J Cell Sci* 119, 314 - 325.

Sheridan, C., Kishimoto, H., Fuchs, R.K., Mehrotra, S., Bhat-Nakshatri, P., Turner, C.H., Goulet, R., Jr., Badve, S., and Nakshatri, H. (2006). CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* 8, R59.

Tseng, D., Volkmer, J.P., Willingham, S.B., Contreras-Trujillo, H., Fathman, J.W., Fernhoff, N.B., Seita, J., Inlay, M.A., Weiskopf, K., Miyanishi, M., *et al.* (2013). Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. *Proc Natl Acad Sci U S A* 110, 11103-11108.

Visvader, J.E., and Lindeman, G.J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 8, 755-768.

Zhang, H., Lu, H., Xiang, L., Bullen, J.W., Zhang, C., Samanta, D., Gilkes, D.M., He, J., and Semenza, G.L. (2015). HIF-1 regulates CD47 expression in breast cancer cells to promote evasion of phagocytosis and maintenance of cancer stem cells. *Proc Natl Acad Sci U S A* 112, 28.

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