ISOLATION AND COMPARISON OF TUMORIGENICITY OF DIFFERENT CELL POPULATIONS FROM THE MCF-7 BREAST CANCER CELL LINE BASED ON CD44 AND CD24 MARKERS

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SUMMARY

Breast cancer stem cells are the origin of breast tumour. Cells expressing marker $CD44^+CD24^{-/dim}$ were considered breast cancer stem cells in human being. The aims of this study is to isolate potential candidate breast cancer stem cell from the MCF-7 cell line to find out whether it is possible to isolate cancer stem cells using two cell surface markers: CD44 and CD24. Stem-like properties were studied *in vivo* by observing the tumorigenicity of different cell populations in immunosuppressed mouse models. MCF-7 cells were subculture for several passages and were sorted out into 3 different cell populations by 2 specific antibodies which bind to CD44 and CD24, using flow cytometry. Cell sub-populations: $CD44^+$, $CD44^+/CD24^+$ and $CD44^+/CD24^{-/dim}$ were further cultured and were then analysed using flow cytometry again to determine the purity of the population. Different cell sub-populations were then harvested and injected into mouse models at two specific doses, 10^3 and 10^6 cells. Tumour formation in the mice was monitored closely and the percentages of mice which have tumour were recorded as results. The results showed that the capacity of causing tumour in mouse was highest in $CD44^+CD24^{-/dim}$ cell population and lowest in $CD44^+$ cell population. These results confirmed $CD44^+CD24^{-/dim}$ cells were the strongest cell population causing tumour in breast cancer cells MCF-7.

Keywords: *Breast cancer cells, MCF-7 cell line, Breast cancer stem cells, Tumorigenicity*

INTRODUCTION

The cancer stem cell model is a concept which proposes that tumours, like normal tissues, are organised in a cellular hierarchy, in which 'cancer stem cells' are the only cells with unlimited proliferation potential and therefore capable of driving tumour growth and metastasis (Reya *et al.*, 2001). To consider cells as cancer stem cells, clonally derived cells from a tumour specimen have to show several characteristics. They must be self-renewal and proliferate; able to differentiate and express markers that are typical of end terminal cells; must be able to generate tumours in animal models that resemble the original tumour from patients after in vivo transplantation. It is believed that cancer stem cells originated from normal stem cells, as they have a longer lifespan than differentiated cells. This allows normal stem cells to accumulate mutations, causes unregulated cell proliferation (Martínez-Climent *et al.*, 2006).

Breast cancer is the fifth most common cause of cancer death worldwide and is the most common cancer in women. Therefore the demand for newer and better treatments for the disease is high and urgent. By eliminating cancer stem cells specifically, which is the source for cancer growth, it can minimise the need for surgery and chemotherapy. Cancer stem cells are present in a very small proportion of the tumour, hence it is believed that conventional chemotherapies could only kill differentiated or differentiating cells, which form the bulk of the tumour but are unable to generate new cells. The population of CSCs could remain untouched and cause a relapse of the disease. Studies have shown that cancer stem cells had been resistant to irradiation treatment and chemotherapy drugs such as epirubicin (Dean *et al.*, 2005; Dave, Chang, 2009). It is an anthracycline drug that acts by intercalating DNA strands, causing complex formation which inhibits DNA and RNA synthesis. Thus novel methods have to be researched to target cancer stem cells specifically, by aiming at their proliferation and growth pathways. Inhibiting these pathways may in turn stop their rapid rate of tumour generation.

Breast cancer stem cells can be identified by their cell surface markers. Common identification method is using $CD44^+/CD24^{-/dim}$Lineage– phenotype, to identify breast tumour initiating cells

(Honeth *et al.*, 2008). This tumour initiating phenotype was proposed by Clarke and colleagues (Al-Hajj, Clarke, 2004), who provided the first proof of principle for the existence of cancer stem cells in solid tumours. Their study showed that in nine breast cancer samples, a minority of cells bearing the surface markers $CD44^+/CD24^{-/dim}$Lineage– were capable of generating tumours in NOD/Immunodeficiency mice even when implanted in low numbers. By contrast, the other cancer cell populations, such as $CD44^+/CD24^+$ failed to generate tumours even when implanted in high numbers. CD44 protein is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. These biological properties are essential to the physiological activities of normal cells, but they are also assisting in the pathologic activities of cancer cells (Miletii-Gonzalez *et al.*, 2005). CD24 is a cell membrane surface proteins linked via glcosyl-phosphatidylinositol. It is expressed in many types of solid tumours, such as leukemias and all type of B cells. It is referred as a B cell-specific marker expressed during early stage of B cell development. Several studies have reported that CD24 expression is a prognostic marker for some solid tumours, such as lung, stomach, prostate and breast tumours etc (Ahmed *et al.*, 2004; Sagiv *et al.*, 2008; Yang *et al.*, 2009). In addition, CD24 was reported to be involved in cell adhesion and metastatic tumour spread by activation of integrin and by stabilization and phosphorylation of focal adhesion kinase (Baumann *et al.*, 2005).

So that, this research will isolate 4 sub-populations in MCF-7 breast cancer cell line: $CD44^+$, $CD44^+CD24^+$, $CD44^+CD24^{-/dim}$ and MCF-7 by flow cytometry and then investigate the tumourigenicity of them *in vivo*.

MATERIALS AND METHODS

Sorting MCF-7 cells based on surface markers CD44 and CD24

Preparation of single cell suspension

All medium and chemicals were pre-warmed to 37°C using water bath. Used medium was removed from the flask and discarded. Monolayer of cells was washed twice with PBS. Trypsin-EDTA was then added (4 mL for T-75 flask, 1 mL for T-25 flask, Nunc, Germany). Flask was then incubated for approximately 10 minutes, until all cells have rounded up. Flask was gently tapped to dislodge the cells from the surface. DMEM/F12 medium supplemented 10% FBS was added to inhibit trypsin activity (8 mL for T-75, 2 mL for T-25). Cell suspension was gently triturated and was then transferred to a 50 mL Falcon tube. Tube was centrifuged at 2500 rpm for 5 minutes. Supernatant was discarded and cell pellet was resuspended with 3 mL of PBS to wash the cells. Tube was then centrifuged at 2,500 rpm for 5 minutes. Supernatant was discarded and cell pellet was resuspended with 1mL of PBS.

Sorting cells into different cell populations

One mL of cell suspension was aliquoted into a 5 mL polypropylene tube. 20µL of PE-Anti-CD44 and 20µL of FITC-Anti-CD24 was added to the tube. The tube was then incubated in the dark on a shaker for 30 minutes. Stained cells were then sorted using FACSCalibur (BD Bioscience). The centrifuge tubes containing the sorted cells were centrifuged at 5,000 rpm for 5 minutes. Supernatant was discarded and cell pellet was resuspended with DMEM/F12 medium. Cell suspension was then transferred to a T-25 flask and observed under an inverted microscope.

Determine purity of cell populations

Used medium was discarded and monolayer of cells was washed twice with PBS. Trypsin-EDTA was then added (4 mL for T-75 flask, 1 mL for T-25 flask). Flask was then incubated for approximately 10 minutes, until all cells have rounded up. Flask was gently tapped to dislodge the cells from the surface. After all cells have rounded up, DMEM/F12 medium was added to inactivate trypsin activity. Cell suspension was gently triturated and transferred to a 50 mL Falcon tube and subjected to centrifugation at 2,500 rpm for 5 minutes. Supernatant was discarded and cell pellet was washed with PBS. Cell suspension was centrifuged at 2,500 rpm for 5 minutes. Supernatant was discarded and cell pellet was resuspended with 50 µL of PBS.

Immunosuppression of *Mus Musculus* Var. *Albino* mice

First day, Busulfan was injected into the intra-peritoneal route into each mouse at a dose of 20 mg/kg/100 µL. On the second and third day, Cyclophosphamide was injected intravenously at the tail vein of each mouse at a dose of 50 mg/kg/100 µL. Injection sites were cleaned with 70% ethanol prior to injection. Injection of tumor cells

commenced on the fourth day and cyclophosphamide injection was continued every 4 days after cells transplantation to maintain immunosuppression at a dose of 25 mg/kg.

Transplantation of cell populations to mouse models

Preparation of single cell suspension

Used medium was removed from the flask and discarded. Monolayer of cells was washed twice with PBS. Trypsin-EDTA was then added (4 mL for T-75 flask, 1 mL for T-25 flask). Flask was then incubated for approximately 10 minutes, until all cells have rounded up. Flask was gently tapped to dislodge the cells from the surface. DMEM/F12 medium supplemented with 10% FBS (fetal bovine serum) was added to inhibit trypsin activity (8 mL for T-75, 2 mL for T-25). Cell suspension was gently triturated and was then transferred to a 50 mL Falcon tube. Tube was centrifuged at 2,500 rpm for 5 minutes at 23°C. Supernatant was discarded and cell pellet was resuspended with 3 mL of PBS to wash the cells. Tube was then centrifuged at 2,500 rpm for 5 minutes. Supernatant was discarded and cell pellet was resuspended with 1 mL of PBS.

Injection of cells into mouse models

Hair on the lateral hind leg area and the breast area were shaved. Injection area was swab with 70% ethanol prior to injection. 20 µL of cell suspension was injected into each mouse subcutaneously at the lateral hind leg and at the mammary fat pad.

RESULTS

Purity of three candidate breast cancer stem cells isolated from MCF-7

Purity of CD44+

Purity analysis was done by running the cells through the flow cytometer, FACSCalibur (Biosciences). Cells were stained with PE-Anti-CD44 antibody prior to analysis. Result showed that the purity percentage of cells which have CD44 surface antigen was 59.12%. 40.97% of cells were either negative with CD44 surface antigen or have very weak fluorescent signals.

Purity of CD44+/CD24+

Result from CellQuest Pro software showed that the percentage of cells that have both CD44 and CD24 antigens on their surface is 75.65%, out of 44145 events/cells. 20.07% of cells did not have any fluorescent signals detected, which suggested that these cells may not have CD44 and CD24 antigens on their membrane surface. 2.50% of cells were only positive with CD24 and 1.69% of cells were only positive with CD44.

Purity of CD44+/CD24-

Result showed that percentage of cells that were positive with CD44 but negative with CD24 was 75.80%. 19.81% of cells were both negative with both surface antigens. 4.39% of cells were both positive with CD44 and CD24, and 0% of cells were positive with only CD24.

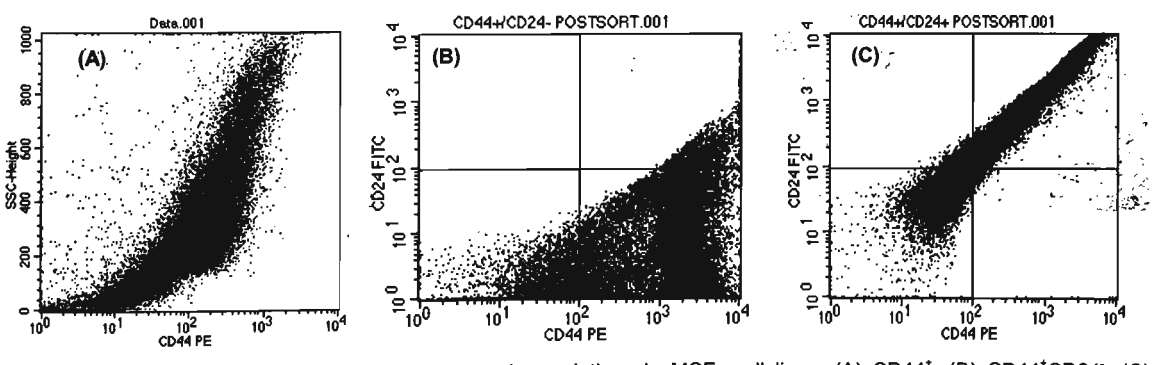

Figure 1. Results of purity confirmation of three sub-populations in MCF- cell lines: (A) $CD44^+$, (B) $CD44^+CD24^-$, (C) $CD44^+CD24^{-/dim}$

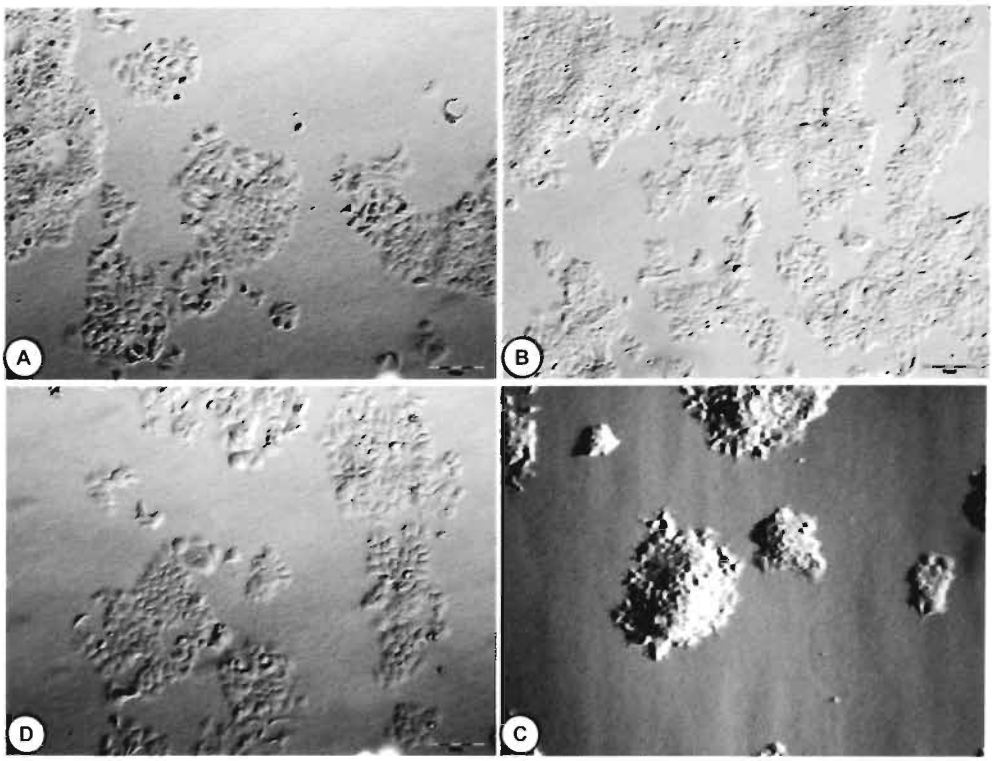

Figure 2. Microscopic view of (A) MCF-7, (B) $CD44^+$, (C) $CD44^+/CD24^+$, (D) $CD44^+/CD24^{-/dim}$ at 100X magnification.

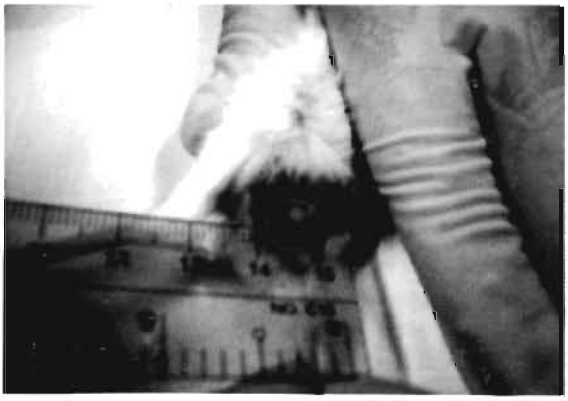

Figure 3. Tumour formed on mouse model. The tumour (in circle) was formed in the leg after injection two weeks. Tumour size was about 2-3 mm in diameter.

Culturing of MCF-7 and its sorted cell populations: Morphology of cells

Under microscopic observation, MCF-7 cells were smaller in size, when compared with the other three cell populations and cells grown very closely together. MCF-7 cells appeared more rounded and grown at a faster rate. Cells could grow above the monolayer when the culture reached confluent and continued to grow into a clump of cells. $CD44^+/CD24^+$ had a similar morphology with MCF-7, but size of cells was slightly larger than MCF-7 cells. In contrast, $CD44^+$ and $CD44^+/CD24^{-/dim}$ cells have a more luminal, elongated shape and smaller. Cells of these populations grow at a much slower rate in culture and will lose its ability to adhere to substrate once the monolayer culture was fully confluent. Cells of $CD44^+$ and $CD44^+/CD24^{-/dim}$ would detach from the surface of the flask and suspended in the culture medium.

Tumor creating capacity of 3 candidate cancer stem cell populations

For each experiment of each different cell populations, 15 mice were used for each dose of

cells, with a total of 30 mice per cell population. Two weeks after tumor transplantation into mouse models, number of mice with tumor growth was recorded. Immunosuppression was maintained in the mouse models throughout the observation period of two weeks using Cyclophosphamide at a dose of 25mg/kg. Result showed that at the dose of 10^3 cells, there was no tumor generated in all the different cell populations. At the dose of 10^6 cells, $CD44^+/CD24^{-/dim}$ cell population has the highest tumorigenicity of 83.3% and $CD44^+/CD24^+$ cell population with the lowest tumorigenicity of 16.7%. $CD44^+/CD24^+$ cell population has an exceptionally low tumorigenicity of 16.7%, which was so much lower than the original MCF-7 cells.

Table 1. Percentage of tumorigenicity of 3 different candidate cancer stem cell populations

Cell population	Dosage of cells	
	% of tumorigenicity (10^3/mouse)	% of tumorigenicity (10^6/mouse)
MCF-7	0%	33.3%
$CD44^+$	0%	50.0%
$CD44^+/CD24^+$	0%	16.7%
$CD44^+/CD24^{-/dim}$	0%	83.3%

DISCUSSION

Purity of $CD44^+$ cell population was only 59.12% and 40.97% of cells were either negative with CD44 surface antigen or have very weak fluorescent signals. This result may not be accurate as errors might have been involved during the experiment which would affect the staining of cells. During the staining procedure, cell suspension was in a very small volume of approximately 50 µL, and the volume of antibody added was 10 µL. Therefore incorrect pipetting of antibody into the polypropylene tube containing the cell suspension might have occurred, which caused inefficient binding of antibody to cell surface antigen.

Second possible error might have been the long incubation period and the presence of light. Cells were stained with antibody and were then incubated for thirty minutes in a near dark environment. Incubation period might have been long, causing the fluorescent signal of the antibody to lose its signal strength. Presence of light might have also affected the fluorescent strength of the antibody, which caused the flow cytometer unable to detect the fluorescent signal and assumed that the cells were negative with CD44 surface antigen.

Third possible error might have been the quantity of antibody. The concentration of cells varied during the experiment, but the quantity of antibody was maintained at 10 µL. The quantity of antibody may not be enough during some of the experiments, causing insufficient antibody to bind with the cell surface antigen.

Another possible reason on the inefficient staining of cells was the clumping of cells. Tumor cells have the tendency to re-clump very easily, which would prevent the antibody from reaching the antigenic sites.

Purity result of other cell populations might also be inaccurate due to these possible errors. If these errors did not occur during the experiments, one main reason that could have explained the low purity of cell populations is that the sorting of cells were done using high recovery option. This caused the flow cytometry to sort the cells less selectively, hence some cells which might not have the required fluorescent signals been also have been sorted out. Second round of cell sorting could be done to increase the purity of cells to > 95%.

Different cell populations which were sorted from MCF-7 had differences in cell morphology. This suggested that the differences in CD44 and CD24 expression could affect the morphology of cells. $CD44^+/CD24^{-/dim}$ cells were clearly more luminal, elongated and smaller in size when compared with MCF-7 cells. This comment was similar to the result of Honeth *et al.* (2008). This might due to the absence of CD24 surface antigen, which caused the cells to be less adhesive and proliferate less rapidly. Therefore $CD44^+/CD24^{-/dim}$ cells grow at a much slower rate than the other cell populations and would detach easily from the flask surface.

Results showed that for the dose of 10^6 cells, $CD44^+/CD24^{-/dim}$ cell population has the highest tumorigenicity, even though these cells proliferate at a slower rate and detach easily from the culture surface. After tumor transplantation into mouse models, palpable tumor was formed after two days.

For $CD44^+/CD24^+$ cell population, the tumorigenicity that was observed in mouse models was the lowest. MCF-7 tumorigenicity was much higher than $CD44^+/CD24^+$, which suggested that cells without CD24 antigen have higher tumor creating capacity. This finding also suggested that the tumorigenicity of MCF-7 and as well as $CD44^+$ cell population were most probably due to the presence of $CD44^+/CD24^{-/dim}$ cells.

For the dose of 10^3 cells, however, no tumor was formed in mouse models for all of the candidate stem cell populations, including MCF-7. In one of the research articles, it was known that $CD44^+/CD24^{-/dim}$ cells have 100% capability of generating tumor in mouse models at the dose of 1000 cells. This may suggests that errors may have occurred during the preparation of cells or the poor condition of the immunosuppressed mice or that the observation duration of 2 weeks was too short for palpable tumor to form. During the serial dilution procedure to obtain the correct dose of 1000 cells, poor pipette technique may have occurred that resulted in lower cell concentration, which may not be enough to generate tumor. Mice may not be effectively immunosuppressed and therefore could not allow cells to grow and replicate as the immune system of the mice would reject the tumor cells. In other research articles, it stated that the observation time for monitoring tumor growth is about 4 to 6 months. Therefore it was clear that the main reason that no tumor was observed in mice with $CD44^+/CD24^{-/dim}$ cells that are known to be highly tumorigenic is due to time constraint. The whole research project duration was given approximately 5 months; therefore more time could not be given to monitor mice for tumor growth.

CONCLUSION

Breast cancer tumor was driven by a small population called breast cancer stem cells. The capacity of breast cancer cell line MCF-7 was low. But if they were enriched with cells expressing $CD44^+CD24^{-/dim}$ cells, they could cause tumor stronger. Expression of CD44 and CD24 protein related with tumor causing capacity. This research confirmed that the $CD44^+CD24^{-/dim}$ cell population was breast cancer stem cells.

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THU NHẬN VÀ SO SÁNH KHẢ NĂNG GÂY KHỐI U CỦA CÁC QUẦN THỂ TẾ BÀO THUỘC DÒNG TẾ BÀO UNG THƯ VÚ MCF-7 DỰA VÀO CHỈ THỊ CD44 VÀ CD24

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TÓM TẮT

Tế bào gốc ung thư vú là nguồn gốc của các khối u vú. Tế bào biểu hiện các chỉ thị $CD44^+CD24^{-/dim}$ được cho là tế bào gốc ung thư ở người. Mục đích của nghiên cứu này nhằm tách các quần thể tế bào gốc ung thư ứng viên khác nhau từ dòng tế bào ung thư vú MCF-7 dựa vào hai marker: CD44 và CD24. Các đặc tính giống tế bào gốc được đánh giá in vivo dựa vào khả năng gây khối u trên chuột suy giảm miễn dịch. Tế bào MCF-7 được cấy chuyền vài thế hệ và được tách thành 3 quần thể phụ khác nhau dựa vào khả năng gắn với 2 marker CD44 và CD24 dựa vào máy flow cytometry. Các quần thể phụ $CD44^+$, $CD44^+CD24^+$, $CD44^+CD24^{-/dim}$ được nuôi cấy tiếp tục để đánh giá và phân tích sử dụng flow cytometry xác định tính tinh sạch của quần thể tế bào. Các quần thể phụ khác nhau được thu nhận và tiêm vào chuột suy giảm miễn dịch theo 2 liều là 10^3 và 10^6 tế bào/con. Sự hình thành khối u được theo dõi và tính phần trăm số chuột xuất hiện khối u. Kết quả cho thấy khả năng gây khối u ở chuột cao nhất ở $CD44^+CD24^{-/dim}$ và thấp nhất ở quần thể $CD44^+$. Kết quả này khẳng định rằng tế bào với kiểu hình $CD44^+CD24^{-/dim}$ là quần thể tế bào gây khối u mạnh nhất và là quần thể tế bào gốc ung thư trong dòng tế bào ung thư vú MCF-7.

Từ khóa: *Dòng tế bào MCF-7, gây khối u, tế bào ung thư vú, tế bào gốc ung thư vú*

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