Novel triple-positive markers identified in human non-small cell lung cancer cell line with chemotherapy-resistant and putative cancer stem cell characteristics

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Abstract. Through the specific identification and direct targeting of cancer stem cells (CSCs), it is believed that a better treatment efficacy of cancer may be achieved. Hence, the present study aimed to identify a CSC subpopulation from adenocarcinoma cells (A549) as a model of non-small cell lung cancer (NSCLC). Initially, we sorted two subpopulations known as the triple-positive ($EpCAM^+/CD166^+/CD44^+$) and triple-negative ($EpCAM^-/CD166^-/CD44^-$) subpopulation using fluorescence-activated cell sorting (FACS). Sorted cells were subsequently evaluated for proliferation and chemotherapy-resistance using a viability assay and were further characterized for their clonal heterogeneity, self-renewal characteristics, cellular migration, alkaline dehydrogenase (ALDH) activity and the expression of stemness-related genes. According to our findings the triple-positive subpopulation revealed significantly higher ($P<0.01$) proliferation activity, exhibited better clonogenicity, was mostly comprised of holoclones and had markedly bigger ($P<0.001$) spheroid formation indicating a better self-renewal capacity. A relatively higher resistance to both 5-fluouracil and cisplatin with 80% expression of ALDH was observed in the triple-positive subpopulation, compared to only 67% detected in the triple-negative subpopulation indicated that high ALDH activity contributed to greater chemotherapy-resistance characteristics. Higher percentage of migrated cells was observed in the triple-positive subpopulation with 56% cellular migration being detected, compared to only 19% in the triple-negative subpopulation on day 2. This was similarly observed on day 3 in the triple-positive subpopulation with 36% higher cellular migration compared to the triple-negative subpopulation. Consistently, elevated levels of the stem cell genes such as *REX1* and *SSEA4* were also found in the triple-positive subpopulation indicating that the subpopulation displayed a strong characteristic of pluripotency. In conclusion, our study revealed that the triple-positive subpopulation demonstrated similar characteristics to CSCs compared to the triple-negative subpopulation. It also confirmed the feasibility of using the triple-positive ($EpCAM^+/CD166^+/CD44^+$) marker as a novel candidate marker that may lead to the development of novel therapies targeting CSCs of NSCLC.

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Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer in the world, which accounts for approximately 80-85% of all lung cancer cases while the remaining 20% is attributed to small-cell lung cancer (1-3). NSCLC is generally resistant to chemotherapy and radiation, which often leads to relapse and even mortality after the initial treatment (4-6). Growing evidence has supported the role of cancer stem cells (CSCs) in the expansion, progression and chemoresistance of tumour cells, including those of lung cancer (7). Current therapies of NSCLC are less likely to affect CSCs, due to the characteristics of these cells or their subpopulations that are intrinsically less sensitive to common treatments (8-10). In addition, CSCs are largely quiescent to treatments, which

allows them to elude standard chemotherapies. Therefore, failure to eradicate these subpopulations are the underlying cause of cancer relapse and fatality (11,12). The effort to identify new markers of lung tumour CSCs that can predict tumour progression through early stage diagnosis is an effective approach, since late detection may significantly lead to higher metastasis and mortality (13). Furthermore, the development of cancer as well as tumour cell metastasis is believed to be driven by CSCs, which has been supported by many studies (14,15). Therefore, in order to increase the efficacy of treatment and detection of the disease, several CSC markers have been identified. Although, many of these markers do exist in the context of lung cancer, nevertheless we believe that a precise characterization of the cells isolated by these markers, specifically for NSCLC is still needed.

Several CSC markers have been identified from various tumours including malignant cells derived from breast (16), pancreas (17) prostate (18), colon (19) and head and neck (20) cancer, as well as leukaemia (21). Concerning lung cancer, putative CSCs derived from various surface markers including CD24 (22), CD133 (23), CD44 (24), CD166 (25) and CD326 (EpCAM) (26) have also been extensively studied and characterised. An example is the epithelial cell adhesion molecule known as the EpCAM, mostly expressed in several human carcinomas and in the majority of normal epithelial cells (27). Due to the strong association between the high expression of EpCAM and the progression of tumour cells in NSCLC, it is believed that this molecule would serve as a prominent marker that can distinguish CSCs from non-CSCs in tumour specimens which are mostly consisted of squamous cell carcinoma (28-30). In addition, EpCAM was observed to be an important biomarker due to its ability to detect circulating tumour cells in the blood of lung cancer patients (31). Another example is the leukocyte cell adhesion molecule (ALCAM) or CD166 that was also found to be a robust marker of lung CSCs, based on the high expression detected in most lung cancer samples and the capacity of the isolated cells to exhibit self-renewal capacity and differentiation *in vivo* (32). The chemotherapy-resistant characteristic is also one of the hallmarks that can specifically discriminate a CSC from a non-CSC subpopulation. For instance, a specific tumour subpopulation isolated from breast (33), colon (34) and gastric (35) cancer is believed to be a CSC subpopulation based on the expression of the homing cell adhesion molecule (HCAM) or CD44. The isolated cells positive for CD44 possess the ability for self-renewal and also the characteristic of being resistant to common chemotherapy, indicating the utility of CD44 as a marker for CSC (35). Furthermore, CD44 was also believed to be crucial for initiating and driving NSCLC stem cell mobility and metastasis (36). Hence, the aim of the present study was to identify and characterise a novel CSC subpopulation from the A549 cell line used as a model of NSCLC using a novel combination of three markers, EpCAM, CD166 and CD44, rather than single markers to strengthen the selection of the CSC population.

Materials and methods

Cell culture of NSCLC cell line (A549). The human NSCLC cell line A549, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown and maintained in a complete RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin and were grown at 37˚C in a humidified atmosphere of 5% CO_2. The cells were maintained in a 75-cm^2 tissue cultured flask and were harvested using 0.25% trypsin-EDTA. All culture reagents were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA) unless otherwise stated.

Sorting of triple-positive ($EpCAM^+/CD166^+/CD44^+$) and triple-negative ($EpCAM^-/CD166^-/CD44^-$) subpopulations. The A549 cells were harvested by incubating the cells with 0.25% trypsin and followed by washing with phosphate-buffered solution (PBS) containing 2% FBS. The suspension cells were then labelled with antibodies (CD326/EpCAM-APC; 1:10 dilutions; cat. no. 347200; CD166-PE; 1:10 dilutions; cat. no. 560903; and CD44-FITC; 1:10 dilutions; cat. no. 347943) (BD Biosciences, San Jose, CA, USA). Briefly, the cells were transferred into 75-mm polystyrene round bottom test tubes (BD Falcon; BD Biosciences) and were suspended in PBS (90 μl) added with 2% FBS at a concentration of $1x10^6$ cells/ml. Subsequently, 10 μl of each antibody were added into the cell suspension and were subsequently incubated for 30 min in the dark. The cells were then washed and filtered through a 40-μm cell strainer to obtain a single cell suspension before sorting. The expression of the CSC markers, EpCAM, CD166 and CD44 was analysed and sorted using a Fluorescence Activated Cell Sorter (FACSAria III; BD Biosciences). Gating was used for sorting out triple-positive ($EpCAM^+/CD166^+/CD44^+$) and triple-negative ($EpCAM^-/CD166^-/CD44^-$) population (Fig. 1).

Cell proliferation assay. MTS assay [3-(4, 5-dimethylthiazol-2-yl)-2H-tetrazolium, inner salt] was purchased from Promega (Madison, WI, USA) and was used to quantify the proliferation of both sorted and unsorted A549 cells at different time-points (24, 48 and 72 h). The cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate and were incubated for the appropriate length of time (24, 48 and 72 h) in a humidified 5% CO_2 incubator at 37˚C. Following that, the MTS solution (15 μl) was added to each individual well for further incubation for 4 h. Subsequently, solubilisation solution (100 μl) was added to the cells and absorbance was assessed using Odyssey® Sa Imaging system (LI-COR Biosciences, Lincoln, NE, USA) at 570 nm, using wells without cells as blank. The viability of cells at different time-points was calculated according to the following formula: cell viability (%) = cells (sample)/cells (control) x 100.

Clonogenic assay. Both sorted [triple-positive ($EpCAM^+/CD166^+/CD44^+$) and triple-negative ($EpCAM^-/CD166^-/CD44^-$)] and unsorted A549 cells were seeded in triplicates (1,000 cells/ml) into a 6-well plate containing 2 ml of complete RPMI-1640 medium per well. The sorted cells were kept in a culture at 37˚C in a 5% CO_2 humidified incubator for 14 days. After 14 days, the colonies were observed using an inverted microscope (Olympus, Tokyo, Japan) at x200 magnification and images from 20 fields of view were captured to evaluate, characterise and manually count the different clones, namely, holoclones,

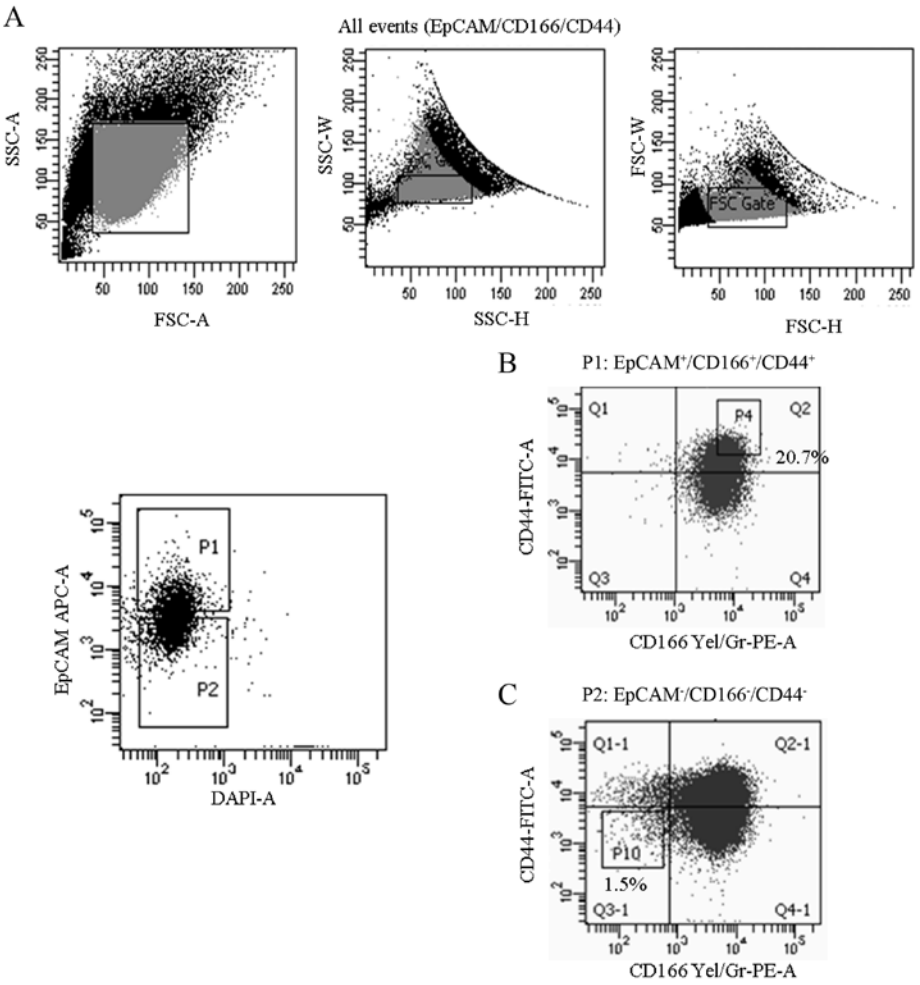

Figure 1. Sorting of triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) and triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) A549 cells. Cells were analysed using the surface markers of EpCAM (CD326)-APC, CD166-PE and CD44-FITC and sorted by FACS. (A) Cell debris and doublets were discriminated to differentiate between viable and dead cells before sorting, as indicated in the first three panels. (B and C) The expression of the triple-positive and the triple-negative subpopulations in A549 cells was 20.7% and 1.5%, respectively.

meroclones and paraclones (Barrandon and Green) (37). For the analysis on the number of colonies formed, the cells were stained using Giemsa (Sigma-Aldrich, St. Louis, MO, USA) and were manually counted. In brief, the supernatant was aspirated and the wells were rinsed twice with PBS, followed by subsequent fixing with 4% formaldehyde for 2 min at 24˚C. Following fixation, the colonies were stained with Giemsa (Sigma-Aldrich) for 15 min in the dark at 24˚C. Following that, the excessive dye was removed and the stained colonies were set to air-dry before analysis and counting.

Spheroid assay. Both sorted [triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) and triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$)] and unsorted A549 cells were suspended in serum-free sphere medium (1.0x10^3 cells/ml) containing DMEM-F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; Life Technologies, Foster City, CA, USA), 1% of B27 supplement (Life Technologies), 20 ng/ml of epidermal growth factor (EGF; Life Technologies), 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies). The cells were re-suspended in a ratio of 1:1 (v/v) of growth factor-reduced Matrigel (BD Biosciences) and serum-free sphere medium. The cells were then seeded in a 96-well ultra-low attachment (ULA) dish (Corning, Inc., Oneonta, NY, USA). The size and number of the formed spheroids were assessed after 20 days of culture by inverted phase contrast microscopy (Olympus).

Chemotherapy-resistance assay. The viability of both sorted and unsorted A549 cells was assessed by MTS assay following treatment with 5-fluouracil (5-FU) and cisplatin. Briefly, the cells were seeded at a concentration of 1x10^4 cells/well in a 96-well plate and were incubated overnight at 37˚C in humidified air containing 5% CO_2. Subsequenlty, 5-FU and cisplatin were added at different concentrations (5, 10, 30, 50, 70 and 90 μM) and incubated for 48 h, and then, 15 μl of MTS solution (CellTiter 96® AQueous One Solution; Promega) was added to each well and further incubated for another 4 h. Solubilisation solution (100 μl) was added to the cells and absorbance at 570 nm was assessed using Odyssey® Sa Imaging System (LI-COR Biosciences). Cell viability was calculated using the formula as aforementioned.

Aldehyde dehydrogenase detection (Aldefluor assay). The Aldefluor assay (StemCell Technologies, Vancouver, Canada) was performed according to the manufacturer's instructions.

Table I. List of primers used in qRT-PCR for the expression of stem cell genes.

Gene	Accession	Forward primer	Reverse primer
REX1	NM_174900	GCGTACGCAAATTAAAGTCCAGA	ATCCTAAACCAGCTCGCAGAAT
SOX2	NM_0031063	CCCCCGGCGGCAATAGCA	TCGGCGCCGGGGAGATACAT
ABCG2	NM_001257386	GTTTATCCGTGGTGTGTCTGG	CTGAGCTATAGAGGCCTGGG
SSEA4	NM_006927	TGGACGGGCACAACTTCATC	GGGCAGGTTCTTGGCACTCT
NANOG	NM_001297698	TCCTCCATGGATCTGCTTATTCA	CAGGTCTTCACCTGTTTGTAGCTGAG
OCT4	NM_001173531	CGACCATCTGCCGCTTTGAG	CCCCCTGTCCCCCATTCCTA

Briefly, a total of $2x10^5$ cells were collected, centrifuged, pelleted and re-suspended in 500 μl of assay buffer before the staining. Aldefluor stain (2.5 μl) was added into the samples followed by the addition of 5 μl ALDH1 inhibitor (diethylaminobenzaldehyde/DEAB) to the control tube. The cells were vortexed and incubated for 30 min at 37˚C in the dark. Subsequently, the cells were centrifuged at 180 x g for 5 min and the collected pellet was re-suspended in 500 μl assay buffer before being subjected to flow cytometry (BD FACS Calibur; BD Biosciences) and analysed using BD CellQuest™ Pro software (BD Biosciences).

Mitochondrial membrane potential ($\Delta\psi_m$) assay. Analysis of mitochondrial membrane potential was conducted using the BD MitoScreen (BD Biosciences). To determine the amount of cells undergoing mitochondrial membrane potential ($\Delta\psi_m$) in response to cisplatin, the cells were incubated in JC-1 stain (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) for 15 min at 37˚C in the dark, and then were washed two times with 1X assay buffer. Analysis on the $\Delta\psi_m$ was conducted based on the intensity of the fluorescence detected in both the FL-1 and FL-2 channels by using a flow cytometer (BD FACSCalibur; BD Biosciences).

Scratch-wound/migration assay. Briefly, both sorted and unsorted A549 cells were seeded at a density of ~$3-4x10^5$ cells/well (6-well plate) in complete RPMI-1640 medium and were grown overnight to 90% confluency. Then, the cells were incubated with colcemid (10 μg/ml) for 2 h for synchronization. After incubation, a scratch was created using a 200-μl pipette tip and the wells were gently washed using PBS to remove any residual debris. Fresh complete RPMI-1640 medium (2 ml) was added followed by incubation for another 48 h. The images of the migrated cells from five fields of view were captured using a relief-phase contrast microscope (Olympus IX71; Olympus) at x400 magnification. The cells migrated into the wound area were evaluated using the following formula: Percentage of cells migrated = [Initial scratch (0 h) - Final scratch (48 h)])/ Initial (0 h) x 100.

Quantitative real time-polymerase chain reaction (qRT-PCR). The mRNA expression of CSC markers in both sorted and unsorted A549 cells was determined by quantitative RT-PCR (qRT-PCR) using a LightCycler 480 (Roche, Mannheim, Germany). Initially, the total RNA was extracted from both cells using an RNAeasy extraction kit (Qiagen, Hamburg, Germany) according to the manufacturer's protocol. Subsequently, cDNA was synthesised from 1 μg of total RNA using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Mannheim, Germany). The qRT-PCR reaction was prepared using a SYBR 1 Master Mix (Roche) as well as different sets of primers (R*EX1, SOX2, ABCG2, SSEA4, NANOG, OCT4* and *GAPDH*) as displayed in Table I. The PCR reactions were run under the following cycle conditions; a pre-denaturation step for 4 min at 95˚C followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. The basic relative gene expression (RQ) was calculated using the ΔΔCt formula and the efficiency (E) of a primer binding equal to 2.

Statistical analysis. Results are expressed as the means ± standard deviation (SD) of three independent experiments. Statistical analysis was performed using the IBM SPSS statistics version 21 (IBM Corp., Armonk, NY, USA). Comparisons between two groups were performed using the two-tailed t-test; P-values <0.01 were considered to indicate statistically significant differences. Comparisons among groups were performed using one factor analysis of variance (ANOVA) followed by the Tukey's post hoc test.

Results

Expression of triple-positive ($EpCAM^+/CD166^+/CD44^+$) and triple-negative ($EpCAM^-/CD166^-/CD44^-$) subpopulations in the A549 cell line. To identify the subpopulation believed to exhibit the putative CSC characteristics, we analysed and sorted subpopulations in the A549 cell line that expressed all three stem cell surface markers (EpCAM, CD166 and CD44), namely the triple-positive ($EpCAM^+/CD166^+/CD44^+$), and the triple-negative ($EpCAM^-/CD166^-/CD44^-$) as controls (Fig. 1). Our findings indicated that the expression of EpCAM, CD166 and CD44 markers in the A549 cell line that was positive for all three markers ($EpCAM^+/CD166^+/CD44^+$) was 20.7% (Fig. 1B), compared to only 1.5% concerning the expression of the triple-negative ($EpCAM^-/CD166^-/CD44^-$) subpopulation (Fig. 1C). Both the triple-positive ($EpCAM^+/CD166^+/CD44^+$) and triple-negative ($EpCAM^-/CD166^-/CD44^-$) subpopulations were sorted from the parental A549 cell line and culture-expanded for further downstream analysis.

Proliferation activity of sorted and unsorted A549 cells. Higher density of cells was observed for both the unsorted

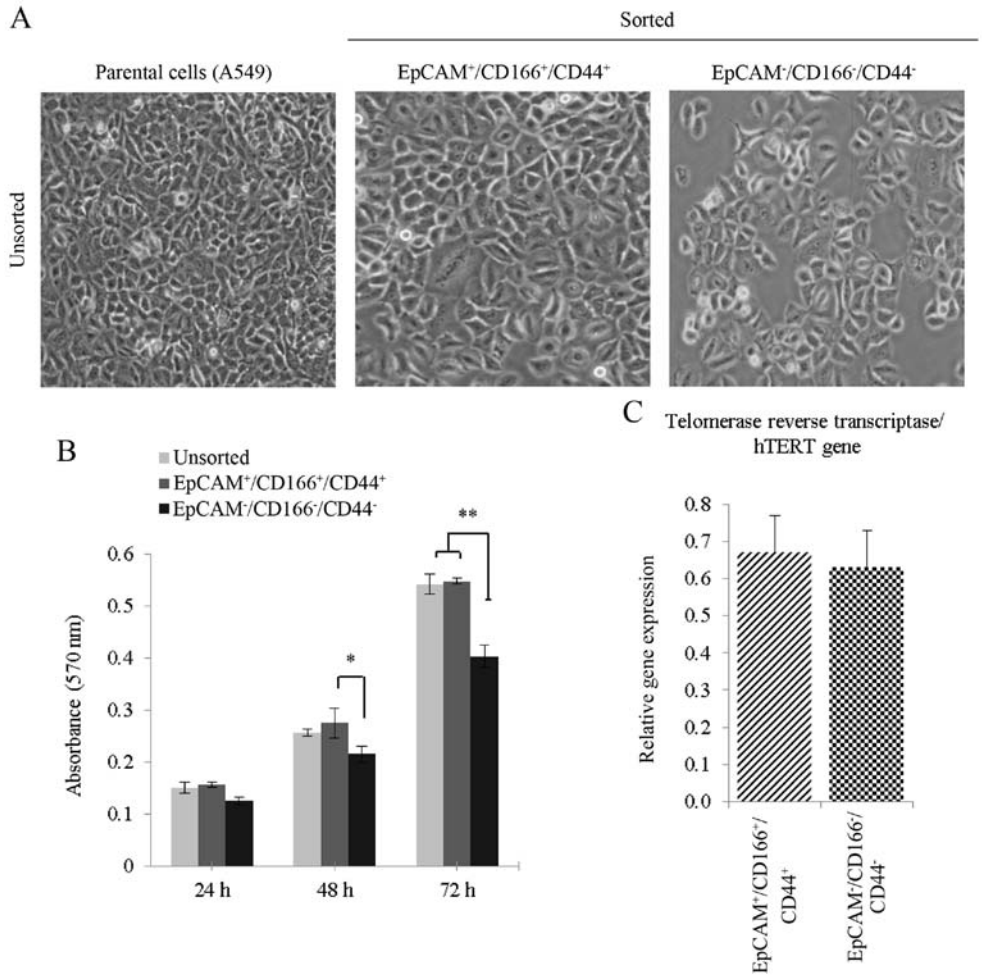

Figure 2. Cell proliferation activity of triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) and triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulations in the NSCLC A549 cell line. (A) The cells were cultured in a 96-well plate at a density of 1x10^3 cells/well and the proliferation activity was assessed by absorbance at 570 nm. Images were captured showing higher number of cells in both the unsorted and triple-positive compared to the triple-negative subpopulations at 72 h post incubation. (B) The triple-positive subpopulation exhibited significantly higher absorbance reading between 48 and 72 h compared to the triple-negative subpopulation indicating higher proliferative activity of these cells. (C) Analysis of the *hTERT* resulted in slightly higher expression of the gene in the triple-positive subpopulation, however there was no significant difference noticed between both subpopulations (*P<0.01, **P<0.001; t-test).

and triple-positive subpopulation as compared to the triple-negative subpopulation (Fig. 2A). Based on the MTS activity, the triple-positive subpopulation (EpCAM$^+$/CD166$^+$/CD44$^+$) demonstrated higher cellular proliferative activities compared to the triple-negative subpopulation (EpCAM$^-$/CD166$^-$/CD44$^-$) between 48 and 72 h (Fig. 2B). In addition, the proliferative activity of the unsorted cells, which comprised of a heterogeneous population was almost similar to that of the triple-positive subpopulation (Fig. 2B), indicating that the triple-positive subpopulation is the main contributor to the cell proliferation activity. Analysis on the *hTERT* gene expression between both the triple-positive and negative subpopulations revealed an equal hTERT activity indicating high proliferative activity in both subpopulations (Fig. 2C).

Triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation reveals compact clone formation as an indicator of 'stemness'. The results revealed that the formation of holoclones was significantly higher (P<0.05), with 75.5±7.8 clones identified in the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation, compared to only 23.0±11.3 clones in the triple-negative subpopulation and 41.5±12.0 clones in the unsorted cells (Fig. 3). Notably, we noticed that the triple-negative subpopulation presented with substantially higher meroclones with 65.5±10.6 clones detected, compared to the triple-positive subpopulation and unsorted cells with only 23.0±1.4 clones and 33.5 ±16.3 clones detected, respectively (Fig. 3).

Triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation demonstrates the ability to form spheres. The study was further validated through the investigation of the self-renewal capability of the sorted cells using a spheroid formation assay. Bigger spheroids reaching an average diameter of 142.7±31.6 μm in size were found in the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation (Fig. 4A). In addition, there were smaller spheroids in the unsorted and the triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulation corresponding to an average spheroid diameter of 112.6±30.7 and 63.0±23.2 μm, as depicted in Fig. 4B. Although both the unsorted and the triple-negative subpopulations were able to form spheroids, which indicated the self-renewal capability of these cells, these characteristics were more noticeable in the triple-positive subpopulation which developed larger sizes of formed spheroids.

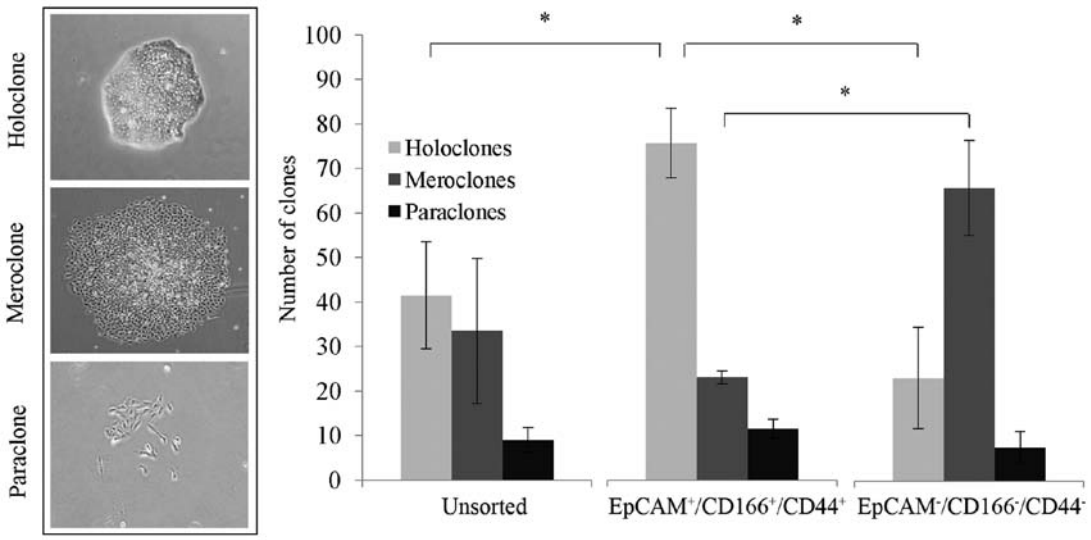

Figure 3. Comparison of clonal heterogeneity (holoclones, meroclones and paraclones) between the unsorted and sorted subpopulations of A549 cells. Cells were seeded at a density of $1x10^3$ cells/well and grown in a 6-well plate for 14 days. The triple-positive ($EpCAM^+/CD166^+/CD44^+$) cells showed higher number of holoclones signifying the stem cell characteristic of the subpopulation, while the triple-negative ($EpCAM^-/CD166^-/CD44^-$) cells presented with greater number of meroclones indicating that the subpopulation exhibited mostly differentiated CSCs ($^*P<0.01$; t-test).

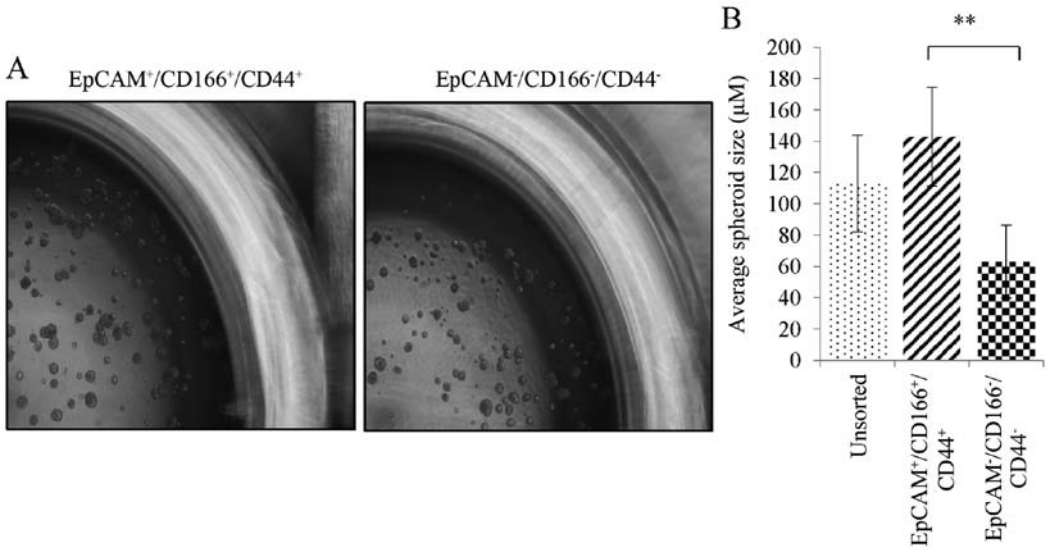

Figure 4. Potential of sorted ($EpCAM^+/CD166^+/CD44^+$ and $EpCAM^-/CD166^-/CD44^-$) subpopulations to form spheroids under serum-free culture condition. (A) Cells were seeded at a density of 500 cells/well in a 24-well ultra-low attachment plate. The triple-positive ($EpCAM^+/CD166^+/CD44^+$) subpopulation formed bigger anchorage independent spheroids compared to the triple-negative ($EpCAM^-/CD166^-/CD44^-$) subpopulation after 14 days of culture. (B) The images showing the sizes of spheres in each well indicated in A, were subsequently analysed as depicted in B. The error bar indicated the average standard deviation of triplicate experiments ($^{**}P<0.001$; t-test).

Chemotherapy-resistant properties of triple-positive ($EpCAM^+/CD166^+/CD44^+$) subpopulation. To investigate the chemotherapy-resistant properties of the triple-positive ($EpCAM^+/CD166^+/CD44^+$) subpopulation, the cells were separately exposed to increasing doses of 5-FU and cisplatin, while both the triple-negative ($EpCAM^-/CD166^-/CD44^-$) subpopulation and the unsorted cells were used as controls. When the cells were exposed to an increased concentration of 5-FU, the percentage of proliferation in the triple-positive subpopulation was markedly higher ($P<0.01$), compared to both the triple-negative subpopulation and the unsorted cells (Fig. 5A). Similarly, higher proliferation rate in the triple-positive subpopulation was noticed compared to the triple-negative subpopulation upon exposure to increasing cisplatin concentrations (Fig. 5B). Our findings also indicated a significant difference ($P<0.01$) in the IC_{50} values of the triple-positive subpopulation with 876.3±260.7 and 65.3±6.9 μM detected compared to the triple-negative subpopulation with only 9.5±1.2 and 35.6±4.1 μM after exposure of both subpopulations to 5-FU and cisplatin, respectively (Table II).

High ALDH1 activity in the triple-positive ($EpCAM^+/CD166^+/CD44^+$) subpopulation. The chemo-resistance characteristic was further investigated through analysis of ALDH activity on triple-positive ($EpCAM^+/CD166^+/CD44^+$) using the Aldefluor™ kit (StemCell Technologies, Vancouver, BC,

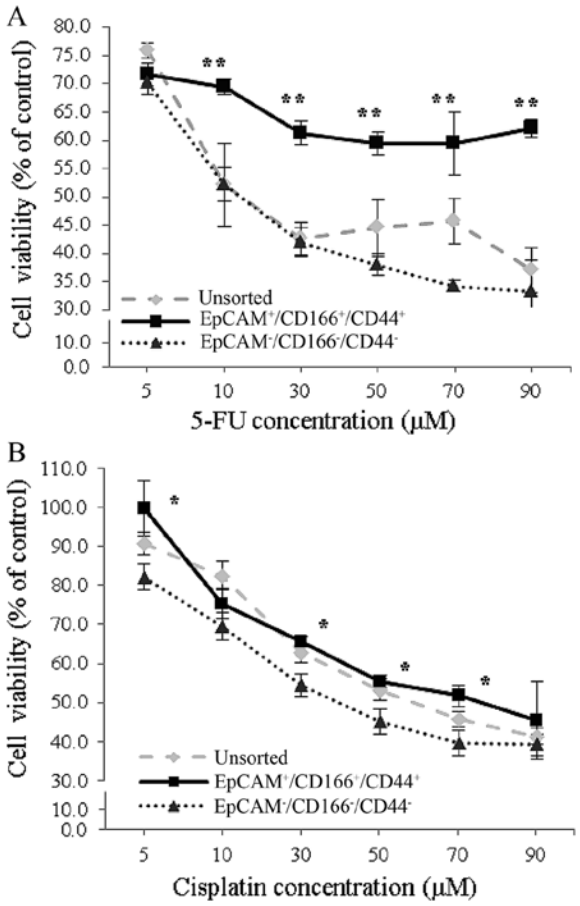

Figure 5. Comparison of cell viability between the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) and triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulations during chemotherapy-induced cytotoxicity. (A and B) The cells were exposed to different concentrations (5-90 μM) of chemotherapy (5-fluouracil, A; and cisplatin, B) for 72 h, followed by analysis of cell viability using Cell Titter-96® AQueous One Solution (Promega). The triple-positive subpopulation exhibited greater chemo-resistant characteristics while the triple-negative subpopulation was more sensitive to the treatments. The error bar indicated the average standard deviation of triplicate experiments (*P<0.01, **P<0.001; t-test).

Canada). Our analysis revealed that the level of ALDH1 activity was significantly higher (P<0.05) in the triple-positive subpopulation with 80.47±1.9% expression level detected, compared to the triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulation (67.88±0.7%) as displayed in Fig. 6. Furthermore, the expression level of ALDH1 in the unsorted cells was 76.26±0.1%, which was almost equal to the expression observed in the triple-positive subpopulation indicating that the ALDH1 activity of the unsorted cells was mostly contributed to the chemo-resistant characteristic of the triple-positive subpopulation (Fig. 6).

Mitochondrial membrane potential ($\Delta\psi_m$) activity of the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation. Chemotherapeutic drugs were able to stimulate changes of the inner mitochondrial membrane and subsequently cause the loss of mitochondrial membrane potential ($\Delta\psi_m$). Using the JC-1 stain, both unsorted and sorted cells were treated with cisplatin and the $\Delta\psi_m$ was subsequently evaluated analysing the JC-1 monomers (green fluorescence) on the FL-2 channel. Our findings indicated that cisplatin treatment resulted in a greater percentage of FL-2 value of 18.9±1.9% as detected in the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation. This was followed by the triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulation and unsorted cells at 8.9±1.0 and 15.1±0.76%, respectively (Fig. 7). Depolarizarion of the $\Delta\psi_m$ indicated a strong activity of the cells undergoing early apoptosis which was detected at a high level in the triple-positive subpopulation.

Table II. IC_{50} values of the A549 cell line subpopulations.

A549 cell subpopulation	IC_{50} values (μM) for the treatments	
	5-fluouracil (5-FU)	Cisplatin
Unsorted	27.8±6.3	59.2±7.9
EpCAM$^+$/CD166$^+$/CD44$^+$	876.3±260.7	65.3±6.9
EpCAM$^-$/CD166$^-$/CD44$^-$	9.5±1.2	35.6±4.1

Results are the mean ± standard deviation (SD). The IC_{50} values were determined as specified in Materials and methods.

Triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation exhibits high epithelial to mesenchymal transition (EMT) characteristics. The following assay was conducted by evaluating the migration ability of the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation compared to the triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulation and unsorted cells. Using an *in vitro* migration model, scratch assay was analysed on day 2 and 3 (Fig. 8). The triple-positive subpopulation demonstrated significantly higher migration (P<0.01) with 56.1±1.4 and 87.6±2.5% wound closure detected on day 2 and day 3, respectively. The findings were compared to the triple-negative subpopulation (with only 19.9±1.7% wound closure on day 2 and 51.9±6.3% on day 3) and indicated that the triple-positive subpopulation had a higher EMT activity.

Triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation of the A549 cell line exhibits the profile of stem cell-associated genes. Finally, to further confirm the intrinsic stem cell properties of the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation, the expression of *SSEA4, REX1*, and *ABCG2* was analysed using the quantitative gene analysis. The triple-positive subpopulation exhibited significantly higher (P<0.001) expression of the specific pluripotent gene, *SSEA4* with 4.9±2.8-fold increase observed compared to the triple-negative subpopulation (Fig. 9A). Slight increase (P<0.05) was observed for the specific stem cell gene, *REX1* in the triple-positive subpopulation compared to the triple-negative subpopulation as depicted in Fig. 9B. This event indicated that the triple-positive subpopulation possessed the features of stem cells as evidenced by the expression of markers related to CSC development. However, no significant differences were detected for the *ABCG2* gene expression between the triple-positive and triple-negative subpopulations (Fig. 9C).

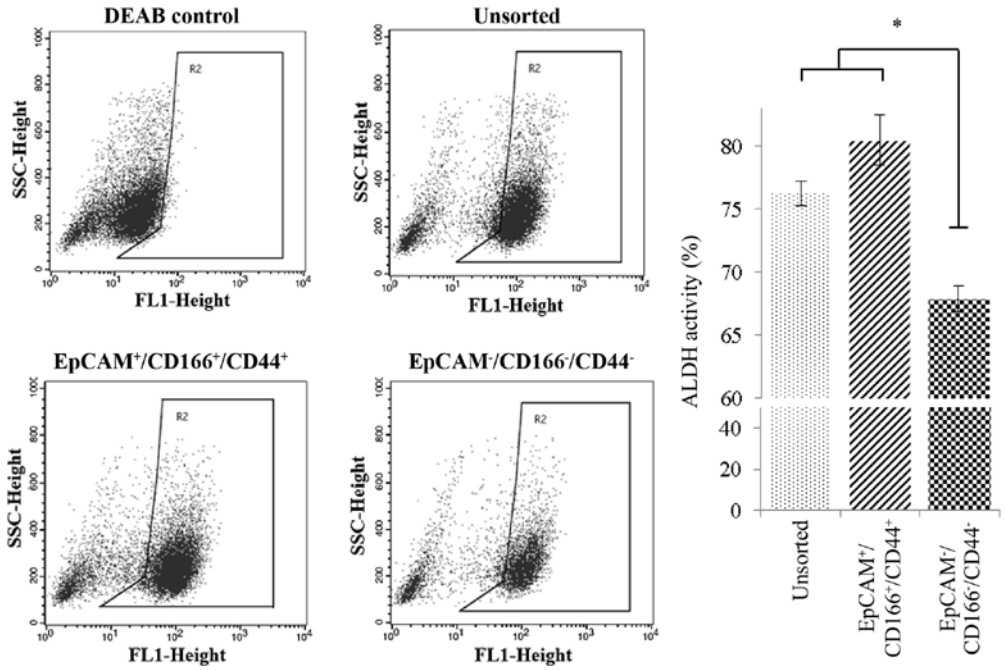

Figure 6. ALDH activity and expression in the sorted triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) and triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulations and unsorted cells derived from the A549 cell line. The triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation presented with higher ALDH activity (80.46%), compared to the triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulation with only 67.88% detected, indicating the resistant characteristic of the triple-positive subpopulation to common treatments and the lower sensitivity of this subpopulation to common chemotherapy. The error bar indicated the average standard deviation of triplicate experiments (*P<0.01, **P<0.001; t-test).

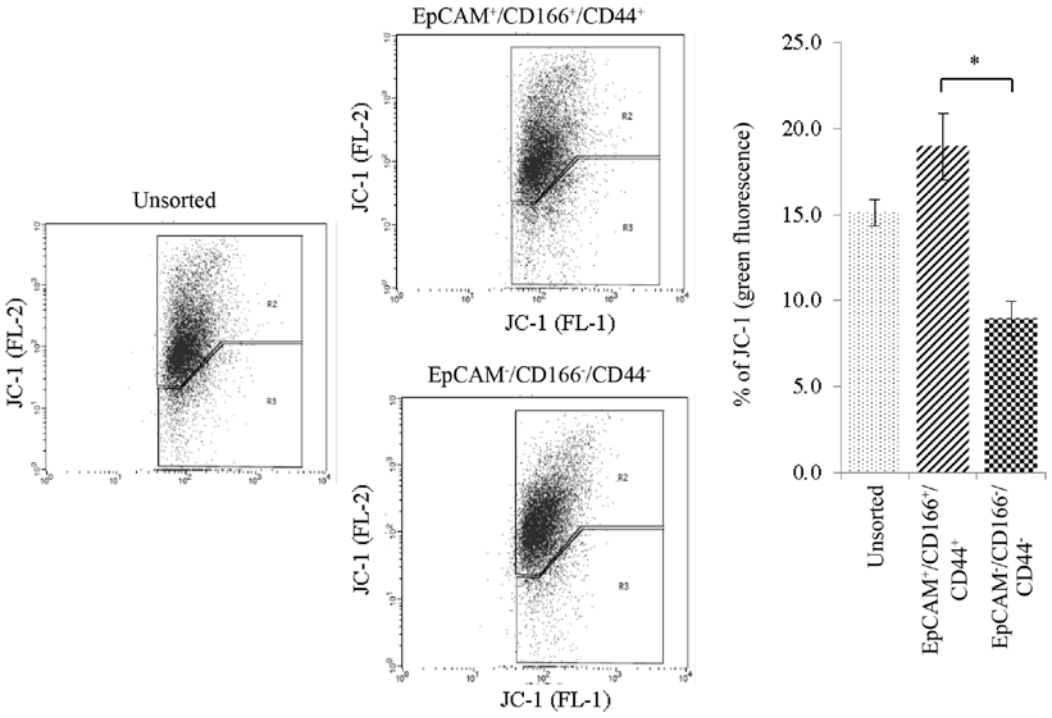

Figure 7. Observation of the mitochondrial membrane potential ($\Delta\psi_m$) in the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) and triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulations prior to treatment with cisplatin. Cells were grown and treated with cisplatin for 72 h and stained with the JC-1 for 15 min at 37˚C in the dark. The fluorescence intensity was detected using flow cytometry and the percentage of the $\Delta\psi_m$ was gated on the R1 area. The error bars indicated the average standard deviation of triplicate experiments (*P<0.01, **P<0.001; t-test).

Discussion

To the best of our knowledge, the present study is the first to investigate and confirm the existence of a novel CSC subpopulation based on the presence of three surface markers, EpCAM, CD166 and CD44 combined, in which these markers can specifically identify and discriminate a distinctive subpopulation of putative CSCs in NSCLC. It has been

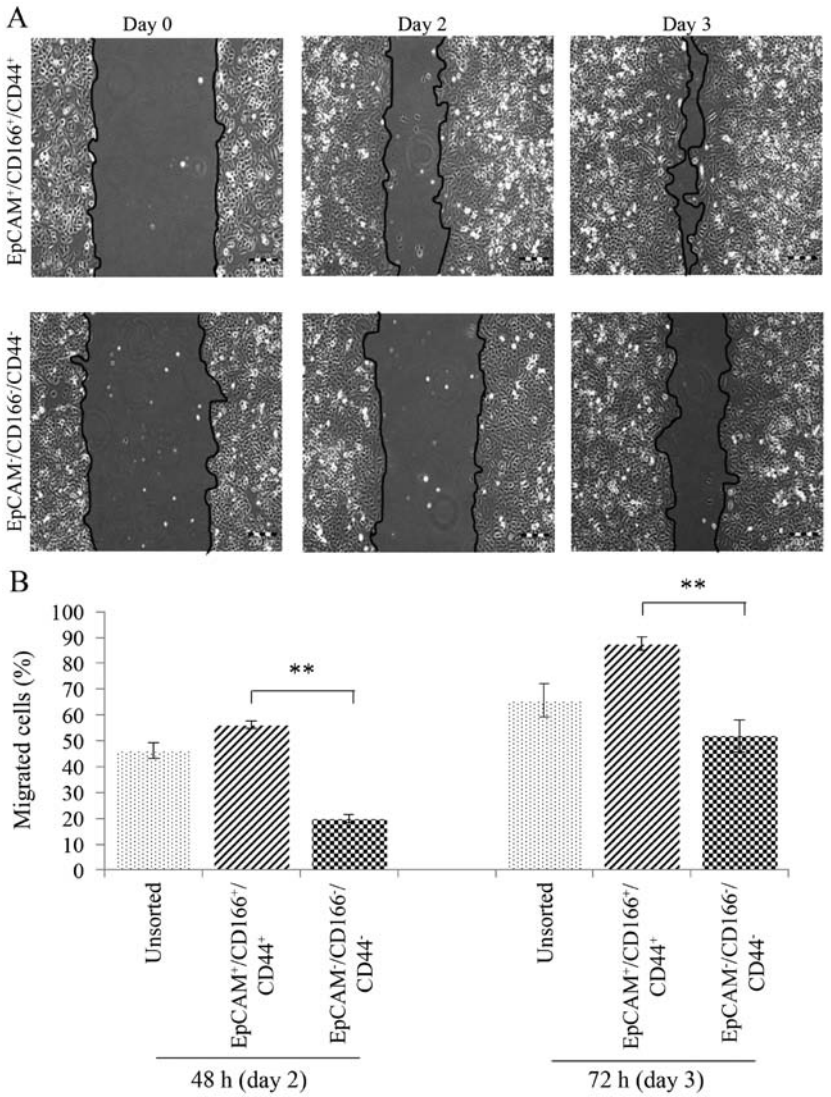

Figure 8. The triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) subpopulation exhibits higher cellular migratory and metastatic potential. (A) Representative photomicrographs and analysis of wound closure were presented for the sorted (EpCAM$^+$/CD166$^+$/CD44$^+$ and EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulations and the unsorted A549 cell line. Higher migratory potential was observed in the triple-positive compared to the triple-negative subpopulation at day 2 and day 3 indicating higher metastatic ability of this subpopulation compared to the control. (B) Wound closures were subsequently analysed as depicted in the graph. The error bar indicated the average standard deviation of triplicate experiments (**P<0.001; t-test).

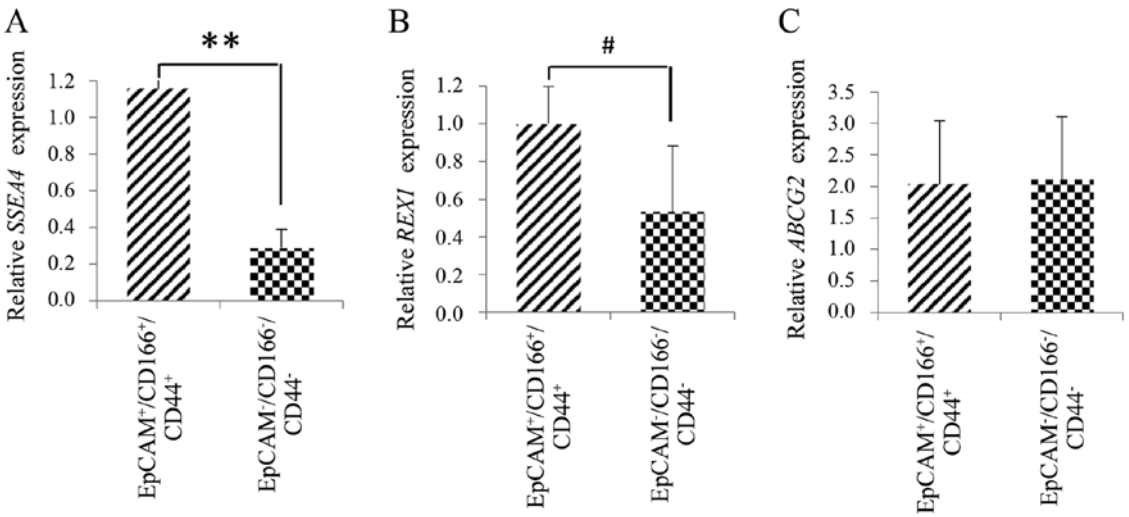

Figure 9. The mRNA expression of pluoripotent and multi-drug resistance genes on both the triple-positive (EpCAM$^+$/CD166$^+$/CD44$^+$) and triple-negative (EpCAM$^-$/CD166$^-$/CD44$^-$) subpopulations. The mRNA expression of (A) *SSEA4*, (B) *REX1* and (C) *ABCG2* was analysed using qRT-PCR and the unsorted cells with *GAPDH* were used as an internal control ($^{\#}$P<0.05, **P<0.001; t-test).

hypothesised that by eliminating this subpopulation, treatment efficacy would be enhanced and chances of tumour relapse reduced. However, the heterogeneity of CSCs is the major impediment that needs to be overcome in order to achieve a better treatment goal. Therefore, a marker that is both robust and specific to identify the major CSC subpopulation is an effective approach for lung cancer treatment. In our previous study, we have identified a potential CSC subpopulation using the combination of two markers from three surface markers (CD166, CD44 and EpCAM) that significantly discriminated CSCs from the non-CSCs in NSCLC (38). Furthermore, we also concluded that these subpopulations ($CD166^+/EpCAM^+$ and $CD166^+/CD44^+$) exhibited the transcriptomic profile of multipotent cells including the self-renewal capacity, ability for adipogenic, as well as osteogenic differentiation and upregulation of the CSC gene pathways, that further confirmed the stemness characteristics of these subpopulations. In addition, the *in vivo* study also confirmed the tumourigenic capacity presented by the ability of these subpopulations to generate bigger tumour bulk, compared to their non-CSCs counterpart ($CD166^-/EpCAM^-$ and $CD166^-/CD44^-$) (38). Therefore, these findings have paved the way to the present study of using a combination of all three markers simultaneously in order to have a stringent selection of CSCs in NSCLC.

Isolating CSCs from the A549 cell line using a combination of all three markers (EpCAM, CD166 and CD44) resulted in two subpopulations known as the triple-positive ($EpCAM^+/CD166^+/CD44^+$) and triple-negative ($EpCAM^-/CD166^-/CD44^-$). In the present study, we found that the triple-positive subpopulation presented with 20.7% expression of the combined markers (Fig. 1), which was higher than the expected percentage of CSCs reported in other studies (39,40). Although the expression was high and may indicate that the combination marker was unspecific, numerous studies have reported the validity of these markers in detecting specific CSC subpopulations, either as single markers, e.g., CD166 (41) and CD44 (42) or co-expressed with other known CSC markers such as CD90 (43) and CD133 (44). Our findings indicated that the triple-positive markers, although highly expressed in the A549 cell line, may serve as specific and yet robust markers that can discriminate a large population of lung CSCs from the non-CSCs due to their high specificity detected in several tumours.

According to Barrandon and Green (1987) (45) who described different clonogenicity to multiplication characteristics, only colonies known as holoclones (colonies with well-defined border) with cellular characteristics of pluripotent stem cells (i.e., high nuclear to cytoplasmic ratio, prominent nucleolus and small cell size) were capable of extensive proliferation, self-renewal and cellular growth. However, the meroclones and paraclones (colonies without a well-defined border) had a limited proliferation capacity and were incapable of self-renewal. From our findings, we observed that in contrast to the triple-negative subpopulation, the triple-positive subpopulation could give rise to higher number of holoclones (Fig. 3). Furthermore, the triple-negative subpopulation consisted of mostly meroclones, indicating that the subpopulation was predominantly governed by differentiated types of CSCs. We also observed that the triple-positive subpopulation had the ability to form bigger spheroids (Fig. 4) compared to its counterpart. Significance in the size of spheroids between the putative CSCs and non-CSCs by *in vitro* analysis that may also reflex the bulk of tumours in xenografts has been reported as an indicator of CSC stemness in numerous studies. The triple-negative subpopulation may also form spheroids as these cells are also tumour cells, however the observation that they were significantly smaller than the triple-positive subpopulation cells indicated its level of tumourigenicity (46). Collectively, the results indicated that the triple-positive subpopulation has the fundamental characteristic of stemness (Figs. 3 and 4) (47).

In addition to clonogenicity, chemoresistance is also an equally important characteristic that can clearly define a typical feature of CSC (48). Since the expression of ALDH1 was high in the triple-positive subpopulation (Fig. 6), it was plausible that the subpopulation was resistant to both cisplatin and 5-FU treatments when compared to the triple-negative subpopulation (Fig. 5). Furthermore, enriched CSCs such as the triple-positive subpopulation would have a lower sensitivity to common chemotherapy as reflected by the higher cell viability post treatment, compared to the unsorted cells where the sensitivity to chemotherapy was higher due to the existence of non-CSCs in their population (49,50). This finding was in line with previous studies that have revealed a similar observation in $CD133^+$ subpopulation derived from colon cancer cells and pretreated with both oxaliplatin and 5-FU (51). The chemotherapy-resistant activity, as confirmed by gene expression analysis, has recently been reported as a potential marker that can specifically discriminate CSCs from non-CSCs (52-55) in different tumour types (52,53,56) However, our observation in the expression of *ABCG2* mRNA (a multidrug resistance family gene) indicated no differences between the triple-positive and triple-negative subpopulations (Fig. 9). Nonetheless, it is believed that the *ABCG2* may not directly influence the chemoresistant characteristic of this subpopulation. Therefore, screening for several other multidrug resistance genes such as the *ABCG1*, *P-glycoprotein* and *MDR* family were necessary in order to fully characterise the chemo-resistant activity of this subpopulation.

There was higher loss of mitochondrial membrane potential ($\Delta\psi_m$) in the triple-positive subpopulation following cisplatin treatment, than the triple-negative subpopulation indicating that this particular subpopulation was more sensitive to the treatment. The treatment also induced loss of the $\Delta\psi_m$ activity which eventually led to an activation of the intrinsic apoptotic pathways in both subpopulations (57,58). A higher $\Delta\psi_m$ activity in the triple-positive subpopulation following treatment was possibly due to a greater proliferation activity as seen in the triple-positive subpopulation (Fig. 2), all of which may lead to the production of a higher number of proliferative cells for apoptosis. It is also known that due to the cisplatin mechanism of action that binds to DNA, the activity of cisplatin is greater in highly proliferative cells since the inhibition activity of cisplatin only peaks during cellular division and mitosis (59).

In addition to the stemness and high resistance to drug treatments, high metastatic and EMT activity are also part of the hallmarks of CSCs (60). EMT would normally result in the migration of CSCs with invasive characteristics, induced by the transforming growth factor-B (TGF-B) and activation

of hedgehog signalling pathway (61) under a hypoxic condition (62). Our findings clearly indicated that the triple-positive subpopulation exhibited significantly higher migratory phenotype on day 2 and 3 when compared to the triple-negative subpopulation. The EpCAM and CD44 target molecules, that have been reported to regulate cell migration and metastasis through the Wnt/β-catenin signalling pathway (63-66), may be the key reason for cellular motility and invasion that contribute to the characteristics of CSCs, and ultimately tumour metastasis (67).

CSCs preferentially express genes relevant for stemness such as *SOX2, NANOG, REX1, SSEA4* and *OCT4* which have been shown to be important for human embryonic stem cell development (68). The mRNA expression of *REX1* and *SSEA4*, were relatively higher (Fig. 9) in the triple-positive subpopulation, indicating the contribution of these pluripotent genes in the functions and maintenance of CSCs. We believed that these genes were not only important markers that can determine a pure population of CSCs, but also suitable candidate genes for targeted therapy to disrupt the regulation of CSCs for an enhanced treatment efficacy of lung cancer.

In conclusion, we revealed for the first time, that the triple-positive subpopulation derived from the A549 cell line possessed CSC characteristics, including being highly proliferative, having greater clonogenicity, ability for self-renewal through spheroid formation, being chemoresistant, and exhibiting high ALDH activity and migratory potential. To better understand the chemoresistance of this subpopulation, an *in vivo* drug-resistant assay can be performed to further verify the characteristics of this subpopulation. Furthermore, the gene expression analysis confirmed that the triple-positive subpopulation expressed higher pluripotent genes (REX1 and SSEA4) compared to the triple-negative subpopulation. Collectively, the present study indicated that the identified triple-positive ($EpCAM^+/CD166^+/CD44^+$) subpopulation may be a potential candidate of CSCs for tumour samples derived from NSCLC and the population may also represent a significant group of chemoresistant cells that can be used for drug screening and chemosensitivity testing.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

KSF conceived, designed and performed the experiments. MNL performed part of the experiment and edited the manuscript. NZ, NAF and AZAR analysed the data. PB and ZZ provided the reagents and tools. NAS and KSF wrote the paper. BHY and PLM edited and reviewed the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board, Institute for Medical Research (IMR), Malaysia and the Medical Research and Ethics Committee (MREC), Ministry of Health (MOH), Malaysia.

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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