Curcumin improves the efficacy of cisplatin by targeting cancer stem-like cells through p21 and cyclin D1-mediated tumour cell inhibition in non-small cell lung cancer cell lines

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Abstract. Natural compounds such as curcumin have the ability to enhance the therapeutic effectiveness of common chemotherapy agents through cancer stem-like cell (CSC) sensitisation. In the present study, we showed that curcumin enhanced the sensitivity of the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation in non-small cell lung cancer (NSCLC) cell lines (A549 and H2170) to cisplatin-induced apoptosis and inhibition of metastasis. Our results revealed that initial exposure of NSCLC cell lines to curcumin (10-40 μM) markedly reduced the percentage of viability to an average of ~51 and ~54% compared to treatment with low dose cisplatin (3 μM) with only 94 and 86% in both the A549 and H2170 cells. Moreover, sensitisation of NSCLC cell lines to curcumin through combined treatment enhanced the single effect induced by low dose cisplatin on the apoptosis of the double-positive CSC subpopulation by 18 and 20% in the A549 and H2170 cells, respectively. Furthermore, we found that curcumin enhanced the inhibitory effects of cisplatin on the highly migratory $CD166^+/EpCAM^+$ subpopulation, marked by a reduction in cell migration to 9 and 21% in the A549 and H2170 cells, respectively, indicating that curcumin may increase the sensitivity of CSCs to cisplatin-induced migratory inhibition. We also observed that the mRNA expression of cyclin D1 was downregulated, while a substantial increased in p21 expression was noted, followed by Apaf1 and caspase-9 activation in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation of A549 cells, suggested that the combined treatments induced cell cycle arrest, therefore triggering CSC growth inhibition via the intrinsic apoptotic pathway. In conclusion, we provided novel evidence of the previously unknown therapeutic effects of curcumin, either alone or in combination with cisplatin on the inhibition of the $CD166^+/EpCAM^+$ subpopulation of NSCLC cell lines. This finding demonstrated the potential therapeutic approach of using curcumin that may enhance the effects of cisplatin by targeting the CSC subpopulation in NSCLC.

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Introduction

Lung cancer is the second leading cause of cancer-related mortality worldwide, and more than 1.6 million cases are diagnosed every year (1). Tobacco smoking and exposure to environmental carcinogens have been found to be the major risk factors in the development of this disease (2). Most lung cancer patients are diagnosed in an advance stage with an overall survival of five years (1). Despite considerable advances in our knowledge and experience in the treatment of lung cancer patients, our capacity to effectively fight and treat this disease is still limited (3). Treatment of lung cancer patients only manages to reduce the burden of the primary lesion but rarely is effective to completely eradicate the tumour cells which in turn leads to relapse and fatality (2). These facts and limitations highlight the need for the greater understanding of the cellular and molecular events that drive tumourigenesis. Thus, therapeutic strategies can be tailored for better treatment efficacies.

Lung cancer can be classically subdivided into small cell lung cancer (SCLC) and three types of non-small cell lung cancer (NSCLC), which include squamous cell carcinoma, adenocarcinoma and large cell carcinoma (4). The existence of several lung epithelial progenitor cells that initiate diverse lung epithelial subtypes and functions is thought to be responsible for this tumour variety (5). The cancer stem cell (CSC) theory suggests that mutations in the progenitor cells lead to the formation of CSCs resulting in cellular hierarchy and clonal expansion within a tumour (6,7). CSCs are known to share common properties with normal epithelial stem cells

including self-renewal, proliferation and capacity for lineage differentiation (6,8,9). However, CSCs may not necessarily be homogeneous in general as they often evolve subsequently by accumulating additional mutations, which in turn results in a complex clonal heterogeneity (10). CSCs are also believed to be the driving source of the malignant phenotype (resistance to chemotherapy, distant metastasis and relapse) in the primary tumour (11). Therefore, therapies that target chemoresistant tumour cells and distant tumour metastasis, which are characteristic of CSCs, may be an effective and yet powerful treatment strategy to eradicate the primary tumour (12,13).

Curcumin (diferuloymethane), a naturally occurring polyphenol extract from the rhizome *Curcuma longa* (Tumeric), possesses biological activities against many types of tumours (14-18). Curcumin modulates numerous target proteins including transcription factors, receptors, kinases, cytokines, enzymes and growth factors (19). Curcumin was found to downregulate the expression of several drug-resistance proteins such as ATP-binding cassette (ABC) drug transporters, P-glycoproteins and multi-drug resistant (MDR) proteins, which resulted in the sensitivity of tumour cells to chemotherapy (20-22). Pre-clinical studies have shown that curcumin acts synergistically with conventional chemotherapeutic drugs to eradicate resistant lung cancer cell lines (20,23,24). Similar findings with different tumours have also been reported *in vitro* as well as in experimental animal models (25-28). In a human breast cancer xenograft model, administration of curcumin markedly decreased the metastasis of breast tumour cells to the lung and suppressed the expression of vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9) and intercellular adhesion molecule-1, which reduced the invasive and metastatic phenotype of the tumour cells (29). Furthermore, curcumin has been found to be safe when administered at ≤10 g/day in humans, thus reducing the difficulty of reaching an effective dose due to dose-limiting toxicity (30).

The antitumour efficacy of curcumin has also been studied recently, either alone or in combination with other antitumour agents on stem-like cells isolated from several tumours using *in vitro* CSC assays (sphere formation, enzyme activity, side population and cell-surface marker expression) as well as *in vivo* animal models. In breast cancer models, 5 μM of curcumin treatment reduced mammosphere formation by 50%, while complete elimination of mammospheres and reduction in aldehyde dehydrogenase 1 (ALDH) enzyme activity (a selective marker noted in most CSCs) were noted as the concentration of curcumin was increased to 10 μM (31,32). A study conducted by Fong *et al* using an *in vivo* glioma model reported that daily treatment of 5 μM curcumin resulted in the reduction of the side population as analysed by flow cytometry (33). Furthermore, curcumin also reduced the expression of CD133 and nestin (neural stem/progenitor markers) indicating the differentiation of gliomal CSCs that eventually led to deregulation of the self-renewal capability of CSCs (34).

CD133 was recently reported as a promising CSC marker noted in prostate cancer (35-37), brain tumours (38-41), colon cancer (42-44) and hepatic carcinoma (45-48). However, in the context of lung cancer stem cells, the utility of the marker appears limited due to the low expression detected in most lung cancer samples (49,50), and the discrepancy of the findings in regards to CD133 in most studies have questioned the prognostic value of this marker in clinical application (50-52). It is therefore important to identify markers that are commonly expressed in most lung cancer samples; hence it can be applied in a larger fraction of lung tumour samples. Other studies established that CSC markers such as CD326 (EpCAM) and CD166 are more robust compared to CD133 as these markers are highly detected in most NSCLC cancer samples (53,54). Furthermore, $CD166^+/Lin^-$ markers were also found to be prominent in NSCLC patients suggesting the applicability of CD166 as a selective marker for CSCs in NSCLC (54). We previously identified and characterised, based on *in vivo* tumourigenicity, a novel $CD166^+/EpCAM^+$ CSC subpopulation isolated from NSCLC cell lines, and showed that this subpopulation has self-renewal capacity, higher mobility, resistance to apoptosis and exhibits mesenchymal lineage differentiation based on gene expression profiling (55). In the present study, we investigated the anticancer effects of curcumin (either alone or in combination with cisplatin) as a drug sensitiser and metastatic inhibitor on both unsorted and sorted (CD166 and EpCAM) cancer stem-like populations derived from NSCLC cell lines. This study will provide further insight into the potential of using curcumin as a sensitiser of CSCs to cisplatin-induced cell death.

Materials and methods

All of the cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The research protocol was approved by our Institutional Review Boards (Medical Research Ethics Committee/MREC, Ministry of Health, Malaysia).

Cell culture. NSCLC cell lines, A549 (ATCC® CRL-185™) and H2170 (ATCC® CRL-5928™) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin (all purchased from ATCC) and grown at 37˚C in a humidified 5% CO_2 atmosphere. Human lung fibroblast (IMR-90) cells were cultured in MEM-α (1x)-Glutamax medium containing 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin (ATCC). Cells were maintained in T75 tissue culture flasks, and the medium was changed three times a week. Confluent cells were harvested by washing in phosphate-buffered saline (PBS) followed by trypsinisation (0.25% in EDTA) for subculturing. All of the cell lines were purchased from ATCC, and culture reagents were purchased from Gibco-Life Technologies (Grand Island, NY, USA) unless otherwise stated.

Sorting of $CD166^+/EpCAM^+$ and $CD166^-/EpCAM^-$ NSCLC cell populations. The NSCLC cell lines (A549 and H2170) were harvested upon incubation with 0.25% trypsin (Life Technologies, Foster City, CA, USA) and washed with phosphate-buffered solution with 2% FBS. The CD166-PE and EpCAM-FITC (BD Biosciences, San Jose, CA, USA) antibodies were used for CSC identification by flow cytometry. Briefly, cells were trypsinised, counted by a haemocytometer and transferred to 75-mm polystyrene round-bottom test tubes (BD Falcon, NJ, USA) at a cell concentration of

$1x10^6$ cells/ml and subsequently stained with 10 μl of antibodies in the dark at 4˚C. The cells were then washed and filtered through a 40-μm cell strainer to obtain a single-cell suspension before sorting. The expression of cancer stem cell markers (CD166 and EpCAM) was analysed and sorted using FACSAria III (BD, Biosciences). Gating used for the sorting of $CD166^+/EpCAM^+$ (Q2) and $CD166^-/EpCAM^-$ (Q3) NSCLC cell lines is depicted in Fig. 3.

Spheroid assay and self-renewal capacity. Sorted lung tumour cells ($1.0x10^3$ cells/ml) were suspended in serum-free medium containing DMEM F12 (Gibco) supplemented with 10 ng/ml fibroblast growth factor (bFGF), 1% of B27, 20 ng/ml of EGF, 1% antibiotic-antimycotic, (all purchased from Life Technologies) and seeded in an 96-well ultra-low attachment (ULA) dish. Spheroid formation was assessed by light microscopy after 20 days of culture. Self-renewal capabilities were also evaluated by monitoring single cells using the live cell analyser (JuLI™ Br; NanoEnTek, CA, USA).

Preparation of curcumin and cisplatin stock. Curcumin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1 ml DMSO to make a stock solution of 10 mM. The curcumin stock was then diluted in complete RPMI-1640 medium to provide a substock and final working concentrations. Cisplatin (Sigma-Aldrich) was prepared as a 10 mM stock in 0.9% sodium chloride (NaCl) and was diluted in complete RPMI-1640 medium to provide a substock. The solution was filtered through a 0.22-μm membrane, aliquoted and stored at -20˚C until further use.

Inhibitory concentration (IC_{50}) of single treatments (curcumin and/or cisplatin) in the NSCLC cell lines. IC_{50} values for the single treatment with either curcumin and cisplatin of NSCLC cell lines were assessed by the MTS [3-(4,5-dimethylthiazol-2-yl)-2H-tetrazolium, inner salt] assay purchased from Promega (Madison, WI, USA). Tumour cells were plated at a density of $1x10^4$ cells/well in 96-well plates and incubated overnight in humidified air with 5% CO_2 at 37˚C. NSCLC cells were then treated with a working concentration of curcumin (10, 20, 30 and 40 μM) and cisplatin (5, 10, 15, 20 and 25 μM) for 48 h. After a 48-h incubation, 15 μl of MTS solution was added to each well and incubated for another 4 h. Solubilisation solution (100 μl) was later added to the cells, and the absorbance at 570 nm was measured using Odyssey® SA Imaging System (Li-Cor, Lincoln, NE, USA), using wells without cells as the blank. Cell viability was calculated according to the following formula: Cell viability (%) = cells (sample)/cells (control) x 100 and IC_{50} was calculated using log formula.

IC_{50} of curcumin sensitisation prior to cisplatin treatment in the NSCLC cell lines. In order to evaluate the efficacy of curcumin sensitisation prior to cisplatin treatment in NSCLC cell lines, both A549 and H2170 cells were initially sensitised/cultured with different doses of curcumin (10, 20, 30 and 40 μM) for 24 h, followed by low dose cisplatin (<3 μM) for another 24 h. Briefly, the tumour cells were seeded ($1.0x10^5$ cells/well) in 6-well plates and sensitised with different doses of curcumin for 24 h. On the following day, the cells were harvested and seeded again in 96-well plates ($1.0x10^4$ cells/well) with medium containing cisplatin (low dose) for another 24 h. At the end of the experiment, 15 μl of MTS solution was added to each well and incubated for another 4 h. Solubilisation solution (100 μl) was later added to the cells, and the absorbance at 570 nm was measured using Odyssey SA Imaging System, using wells without cells as the blank.

Toxicity of curcumin and cisplatin in the human lung fibroblast (IMR-90) cell line. The IC_{50} values of both curcumin and cisplatin in the A549 and H2170 cells were tested on IMR-90 cells to evaluate the toxic effect of curcumin and cisplatin on normal cells. IMR-90 cells were seeded overnight in 96-well plates at a density of $1.0x10^4$ cells/well in 100 μl complete MEM-α. Subsequently, 100 μl of either curcumin and/or cisplatin (concentration based on IC_{50} of A549 and H2170) was added to the cells and incubated for 48 h. The viability of the IMR-90 cells was assessed by adding 10 μl of Presto Blue (BD Pharmingen, Franklin Lakes, NJ, USA) to each well and incubated for 2 h before the absorbance was measured at 570 nm.

Apoptosis assay. The apoptosis assay was conducted using the Annexin V/propidium iodide (PI) apoptosis kit purchased from BD Pharmingen. In brief, $9.0x10^5$ cells/well of sorted and unsorted NSCLC cells were seeded into 6-well plates and incubated overnight. Direct combination (synergistic effects) of both curcumin and cisplatin on the NSCLC cell lines was performed by incubation of the cells in medium containing the single treatment (cisplatin or curcumin) and combination of both using the IC_{50} doses for 48 h. Indirect combination (sensitising effects) of curcumin was performed by incubating the NSCLC cell lines with curcumin (IC_{50} value) for 24 h, followed by incubation with low dose cisplatin (3 μM) for another 24 h. After treatments for 48 h (synergistic and sensitisation), both NSCLC cell lines were harvested by trysinisation and collected by centrifugation. The cell pellet was suspended in 100 μl of 1X Annexin V binding buffer (Becton Dickinson BD) and 1 μl of Annexin V-FITC was added. Antibody incubation was performed at 4˚C for 20 min, and 1 μl of PI was later added before FACS acquisition. Stained cells were subjected to flow cytometric analysis using a FACSCalibur instrument (Becton Dickinson BD), and a total of 10,000 events were acquired and analyzed using Cell Quest software (Becton Dickinson BD).

Scratch-wound/migration assay. Briefly, sorted and unsorted NSCLC cells were seeded at a density of ~$3-4x10^5$ cells/well in complete medium and grown overnight to a 90% confluent monolayer. The next day, the cells were treated with colcemide (10 μg/ml) for 2 h for cell synchronisation. After incubation, a scratch wound was inflicted using a sterile 200-μl pipette tip and gentle washing was carried out twice using PBS to remove debris. Cells were then incubated with 2 ml of media containing both single treatments (cisplatin and curcumin) and/or the combination of both for another 48 h. The concentration of curcumin and cisplatin (single treatments and/or combination) used for the assay was based on the IC_{50} values evaluated on both A549 and H2170 cells. Images of migrated cells (five fields of each triplicate well) were captured using relief contrast microscopy at x40 magnification (Olympus IX 71; Olympus, Tokyo, Japan) and analysed for 48 h. The

Table I. Human primer sequences used for qRT-PCR.

Gene	Accession	Sense primer	Antisense primer	Product size (bp)
Apaf 1	NM_013229.2	CACGTTCAAAGGTGGCTGAT	TGGTCAACTGCAAGGACCAT	214
Cytochrome *c*	NM_018947.5	GGAGGCAAGCATAAGACTGG	GTCTGCCCTTTCTCCCTTCT	267
Caspase-9	XM_005246014.1	TGTGGTGGTCATCCTCTCTCA	GTCACTGGGGGTAGGCAAACT	331
p21	NM_000389.4	CTCAGAGGAGGCGCCATG	GGGCGGATTAGGGCTTCC	517
Cyclin D1	XM_006718653.1	CGGAGGACAACAAACAGATC	GGGTGTGCAAGCCAGGTCCA	350
GAPDH	NM_001289746.1	TGAAGGTCGGAGTCAACGGATT	CATGTGGGCCATGAGGTCCACCAC	530

Table II. IC_{50} values were determined by proliferation assays as specified in Materials and methods.

	IC_{50} values (μM) for the treatments	
NSCLC cell lines	Curcumin	Cisplatin
A549	40±9.3	30±5.0
H2170	30±8.8	7±0.8

Results are the mean ± standard deviation (SD).

numbers of cells that migrated into the wound area were evaluated using the formula: Percentage of migrated cells = [initial scratch (0 h) - final scratch (48 h)]/initial (0 h) x 100.

Post-treatment effects on CSC marker expression (CD326 and CD166) in NSCLC cells analysed by FACS. NSCLC cells (A549 and H2170) were seeded in 6-well plates at a density of $9.0x10^5$ cells/well and incubated overnight. After incubation, the cells were treated with IC_{50} values of curcumin and cisplatin, respectively for 48 h. Cells were then harvested and washed with ice-cold PBS. Antibodies (CD326 and CD166) (10 μl) were added to each tube and incubated for another 20 min in the dark at 4˚C. The cells were then suspended in ice-cold PBS supplemented with 2% FBS, and a total of 10,000 events were acquired and analysed using Cell Quest software (Becton Dickinson BD).

Quantitative real time-polymerase chain reaction (RT-qPCR). Initially, total RNA was extracted and evaluated for purity as previously described. Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Nonnenwald, Penzberg, Germany) was used to synthesise cDNA according to the protocols recommended by the manufacturer. Quantitative RT-PCR (qRT-PCR) was performed using the Light Cycler 480 (Roche, Mannheim, Germany), on sorted lung tumour cells subsequent to treatment either by single agent (cisplatin or curcumin) or direct combination of both (synergistic effects) based on the IC_{50} values. The qRT-PCR reaction was prepared using SYBR 1 Master Mix (Roche Applied Science, Penzberg, Germany) and primers as stated in Table I. PCR conditions were set under the following cycle conditions: pre-denaturation for 4 min at 95˚C followed by 40 cycles consisting of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec followed by dissociation curve. The basic relative gene expression (RQ) was calculated using the ΔΔCt formula and the efficiency (E) of primer binding equal to 2.

Statistical analysis. All data are expressed as the mean ± standard deviation (SD) of three independent experiments. Comparison between two groups was performed using the two-tailed t-test. P-values of <0.01 were considered to indicate statistically significant differences.

Results

The IC_{50} values of curcumin and cisplatin for both A549 and H2170 cell lines. The IC_{50} values of A549 and H2170 cells treated with curcumin and cisplatin were assessed by MTS assay at 48 h. The results showed that exposure of NSCLC cell lines (A549 and H2170) to a range of curcumin (≤40 μM) and cisplatin (≤25 μM) concentrations resulted in IC_{50} values of 41 and 30 μM and 33 and 7 μM, respectively (Fig. 1A and B). Furthermore, we noted that the IC_{50} values for both A549 and H2170 cells to curcumin were almost equal. However, the IC_{50} value of cisplatin in the H2170 cells was markedly lower compared to that for the A549 cells indicating higher sensitivity of H2170 cells to cisplatin-induced inhibition. Based on the IC_{50} value indicated, the combination of 41 μM curcumin and 30 μM cisplatin was selected for A549 cells and 33 μM of curcumin and 7 μM of cisplatin were selected for H2170 cells for further downstream study (Table II).

Curcumin sensitisation enhances the tumour growth inhibitory effect of low dose cisplatin. To determine whether curcumin sensitises NSCLC cell lines to the tumour inhibitory effect of low dose cisplatin (≤3 μM), NSCLC cells (A549 and H2170) were incubated overnight with different doses of curcumin (10, 20, 30 and 40 μM), harvested and seeded again with low dose cisplatin (≤3 μM) for another 24 h. Treated NSCLC cells were subsequently evaluated for cell viability using the MTS assay. Treatment of the A549 and H2170 cells with curcumin (10-40 μM) markedly enhanced the sensitivity of both NSCLC cell lines to cisplatin (Fig. 2A and B).

Treatment of both A549 and H2170 cells with 3 μM cisplatin alone (low dose) was found to be ineffective to induce inhibition of growth in the NSCLC cell lines (tumour viability ~80%). However, this treatment became highly effective similar to the IC_{50} concentration (tumour viability ~50%) when both A549 and H2170 were initially sensitised to different concentrations

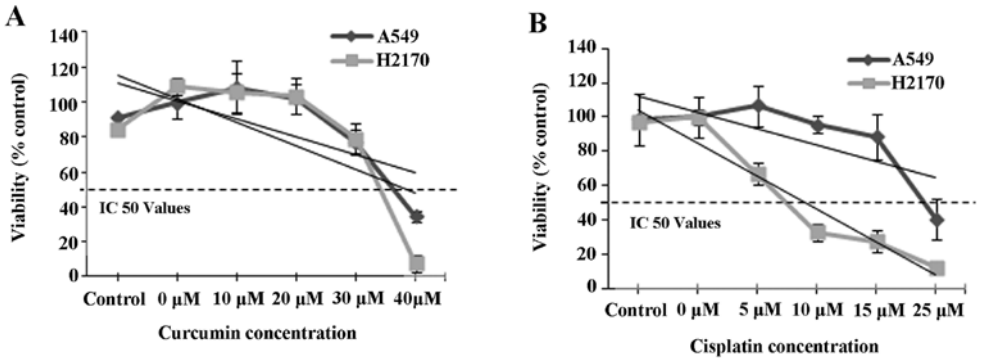

Figure 1. IC_{50} values of curcumin and cisplatin in NSCLC cell lines by MTT assay. The viability of A549 and H2170 cells was measured after a 48-h treatment with the indicated concentrations of curcumin (A) and cisplatin (B).

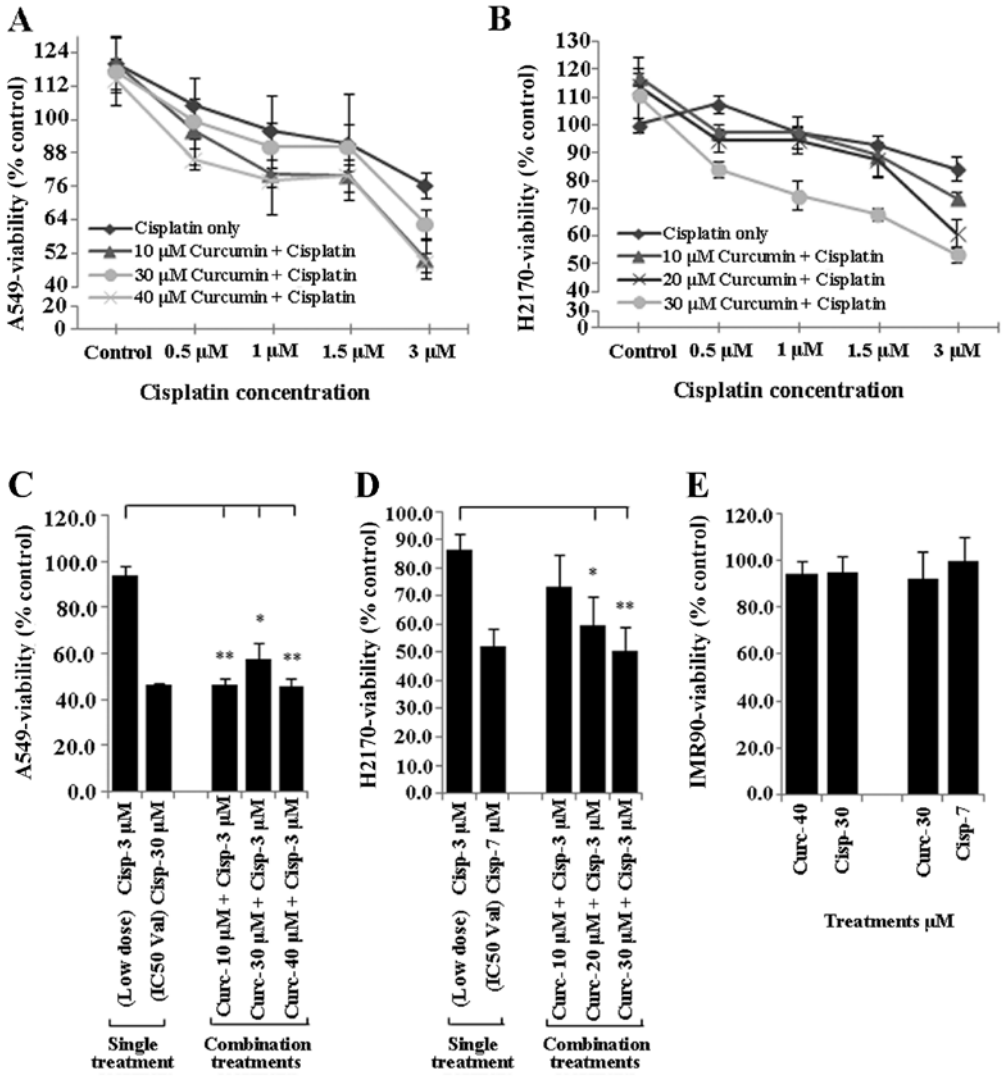

Figure 2. Curcumin sensitisation increases the efficacy of low dose cisplatin. Exposure of NSCLC cell lines (A549 and H2170) with different concentrations of curcumin (10-40 μM) for 24 h prior to treatment with an ineffective low dose of cisplatin (≤3 μM) increased the efficacy of cisplatin on A549 (A) and H2170 (B) cells as the percentage of viability was reduced compared to the basal (cisplatin only) level. Bar diagram showing that cell viability of the NSCLC cell lines was significantly inhibited to an average of ~51.2 and ~54.9% (almost equal to the IC_{50} value) following the combination treatments as compared to the single treatment (cisplatin, 3 μM) in the A549 (C) and H2170 (D) cells, respectively. Moreover, exposure of IMR-90 cells to curcumin and cisplatin caused no growth inhibition (E); *P<0.01, **P<0.001; t-test.

of curcumin (10-40 μM) (Fig. 2C and D). Moreover, exposure of IMR-90 cells to the IC_{50} values of curcumin and cisplatin in both NSCLC cell lines did not induce toxicity to the cells, as the percentage of viability was higher than 90% (Fig. 2E).

Table III. The percentage of subpopulations in NSCLC cells by CSC marker expression post-treatment.

Cell lines	Subpopulations	Treatments		
		Curcumin (%)	Cisplatin (%)	Combination (%)
A549	$CD166^+/EpCAM^+$	25.2±3.2	25.1±7.4	34.6±0.7
	$CD166^-/EpCAM^-$	14.9±3.6	21.4±4.2	23.6±12.6
H2170	$CD166^+/EpCAM^+$	47.4±18.4	50.1±14.3	55.0±0.4
	$CD166^-/EpCAM^-$	1.3±0.2	2.2±0.2	1.7±0.4

Subpopulation of $CD166^+/EpCAM^+$ cells showed higher marker expression at the basal level both in A549 and H2170 cells and were enriched upon the treatments. $CD166^-/EpCAM^-$ cells were selected as a control for $CD166^+/EpCAM^+$. Results are the mean ± standard deviation (SD).

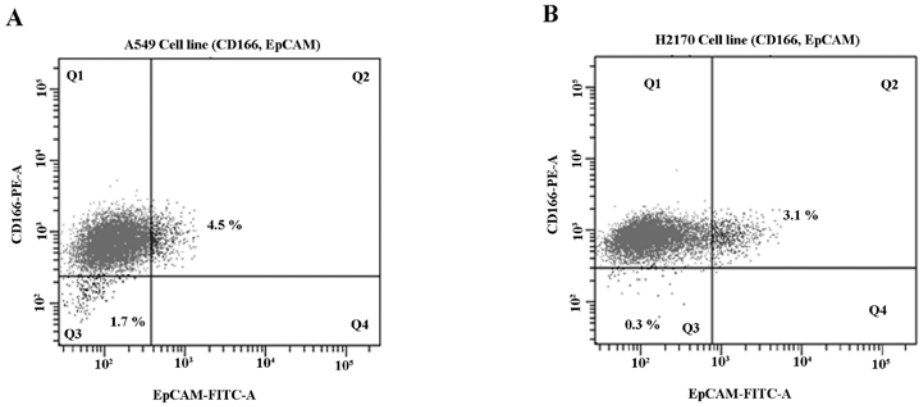

Figure 3. Sorting of double-positive ($CD166^+/EpCAM^+$) and double-negative ($CD166^-/EpCAM^-$) subpopulations in NSCLC cell lines (A549 and H2170). Both NSCLC cell lines were analysed for subpopulations using CD166-PE and EpCAM-FITC and sorted by flow cytometry. A549 cells showed 4.5% (Q2) expression of double-positive and 1.7% (Q3) expression of double-negative subpopulations (A), while H2170 presented with 3.1% (Q2) double-positive and 0.3% (Q3) double-negative subpopulations (B).

Isolation of $CD166^+/EpCAM^+$ and $CD166^-/EpCAM^-$ subpopulations from the NSCLC cell lines. The expression of CSC markers (CD166 and EpCAM) in the NSCLC cell lines was studied and we found a small population of NSCLC cells (A549 and H2170) that showed positivity for CD166 and EpCAM ($CD166^+/EpCAM^+$) (Fig. 3). The NSCLC cells showed consistent double-positive expression of $CD166^+/EpCAM^+$ ranging from 3.0 to 4.5% (Fig. 3). This was consistent with the characteristics of CSCs in a tumour population indicating that the expression of CSC markers should be within ~4% of the total population (56). Moreover, the double-negative ($CD166^-/EpCAM^-$) population was much lower in the NSCLC cell lines with 1.7 and 0.3% in both the A549 and H2170 cells, respectively (Fig. 3A and B). The $CD166^+/EpCAM^+$ and $CD166^-/EpCAM^-$ populations were sorted from the A549 and H2170 cells into a 15-ml tube containing complete medium and transferred to a T75 flask for expansion and further downstream study.

Tumour sphere formation and self-renewal capacity of the $CD166^+/EpCAM^+$ subpopulation. The ability of the double-positive ($CD166^+/EpCAM^+$) subpopulation sorted from the NSCLC cell lines to form three-dimensional spheres in serum-free medium containing stem cell growth factors (EGF and bFGF) on non-adherent plates was examined. The sorted NSCLC cells grew as anchorage-independent spheres after 14 days of culture (Fig. 4B). We observed that the isolated $CD166^+/EpCAM^+$ subpopulations of both A549 and H2170 cells were able to form tumour spheres with an average size ranging from 50 to 200 μm in diameter (Fig. 4B). We also noted that the $CD166^+/EpCAM^+$ subpopulation isolated from A549 cells had the ability for self-renewal and produced daughter cells, which is an important characteristic of CSCs (Fig. 5B). However, there were cells observed as non-dividing (Fig. 5A), which were a dormant phenotype of CSCs.

Combination of curcumin and cisplatin enriches the $CD166^+/EpCAM^+$ CSC subpopulation. In order to study the combination effects of curcumin and cisplatin on the regulation of CSC subpopulations, the expression of two combination markers (CD166 and EpCAM) which were previously described as markers of lung cancer CSCs, were evaluated in the NSCLC cell lines (A549 and H2170) after treatment with either a single agent of curcumin or cisplatin, or a combination of both. Treatment of A549 and H2170 cells with the combination of curcumin and cisplatin led to an average increase of ~10 and ~6% expression of the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation, respectively, as compared to both curcumin and cisplatin treatment alone (Table III). Combination treatment only led to ~2% increase and a slight reduction (~0.5%) in the $CD166^-/EpCAM^-$ subpopulation noted in the A549 and H2170

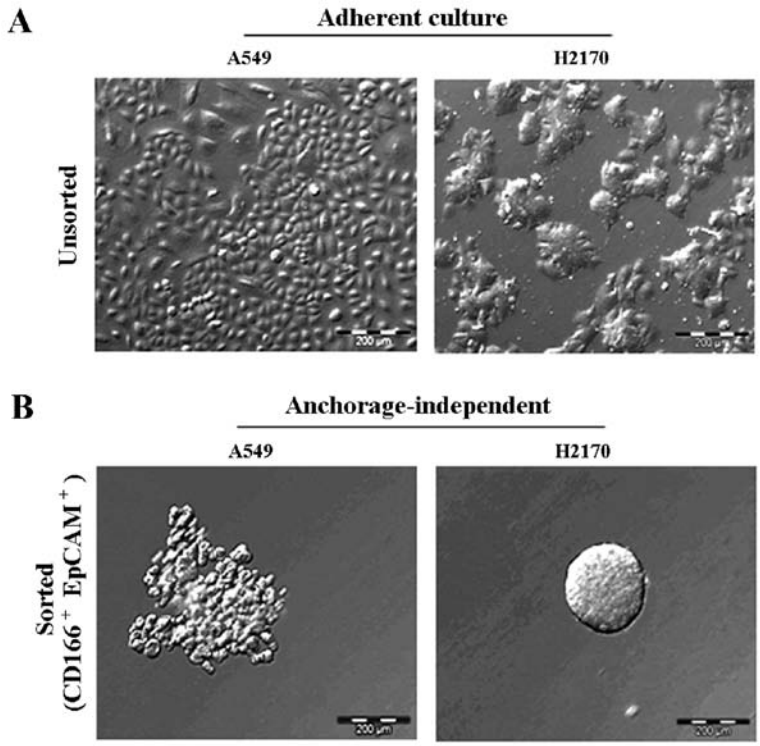

Figure 4. Isolated CSCs from NSCLC cell lines formed anchorage-independent self-renewing tumour spheres. Each of the cell lines exhibits a unique morphology observed as monolayers in the A549 cells, while H2170 as colonies in adherent culture (A). Anchorage-independent spheres formed by CD166$^+$/EpCAM$^+$ cells isolated from both A549 and H2170 cells, 14 days of culture in serum-free stem cell medium (B).

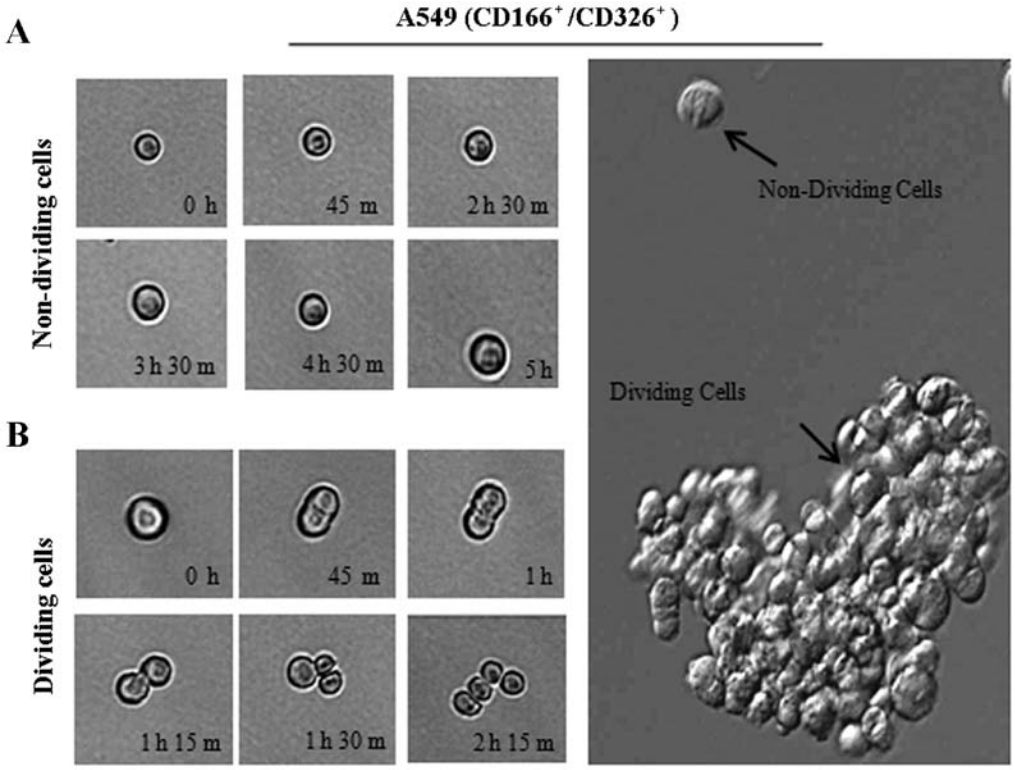

Figure 5. Phase-contrast images of a single CD166$^+$/EpCAM$^+$ cell derived from A549 cells cultured in 96-well plates under anchorage-independent, serum-free conditions. Sorted CD166$^+$/EpCAM$^+$ cells either in the non-dividing phase (A) or actively dividing phase (B) were recorded at different time points as indicated.

cells, correspondingly (Table III), suggesting that the combination of both curcumin and cisplatin synergistically acted to enrich the double-positive (CD166$^+$/EpCAM$^+$) subpopulation as compared to each single treatment alone.

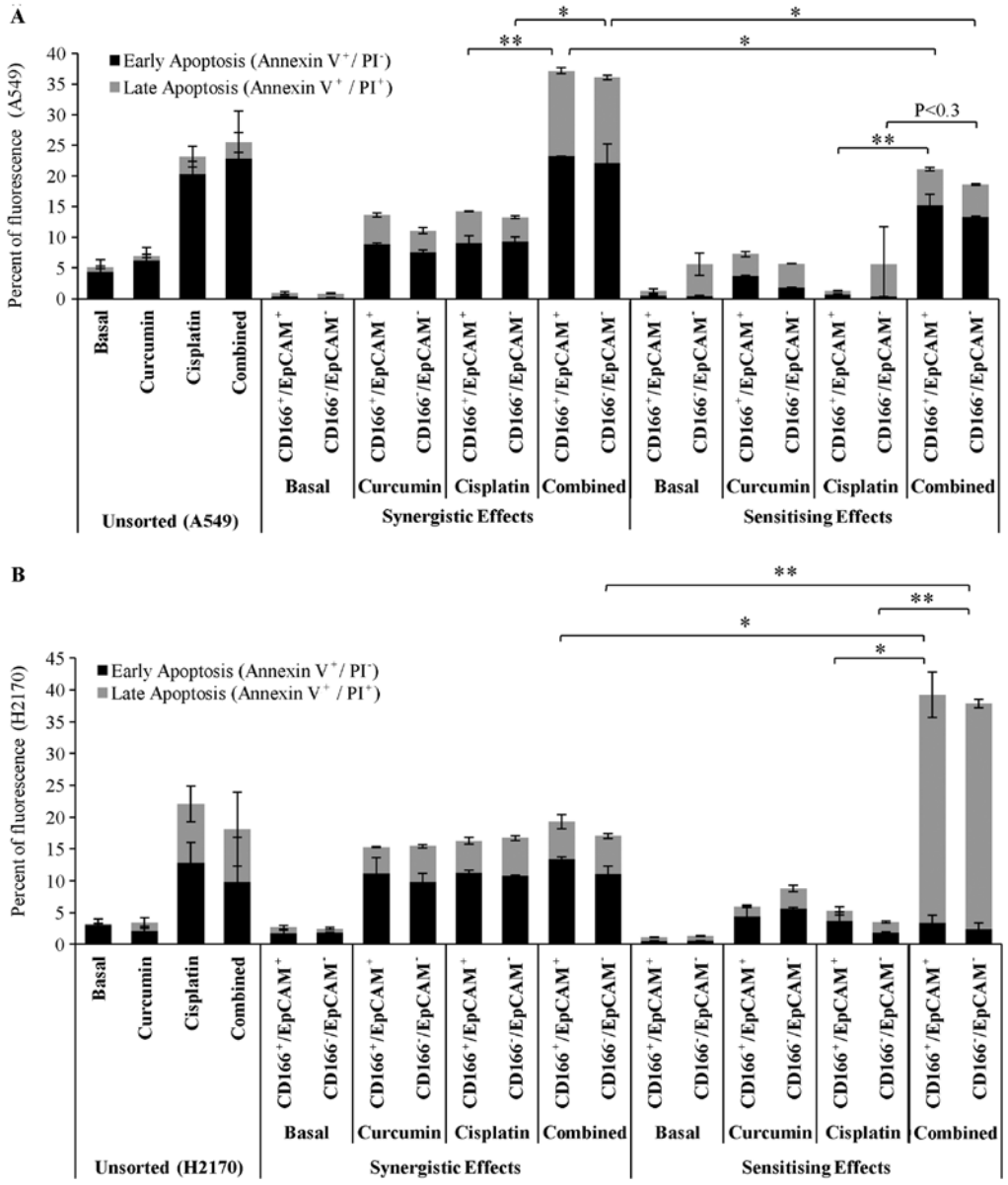

Figure 6. Curcumin improves the sensitivity of the CSC subpopulation ($CD166^+/EpCAM^+$) to cisplatin-induced apoptosis. Both sorted and unsorted NSCLC cells lines were incubated with curcumin either by direct combination (synergistic) with cisplatin or indirect combination (sensitising) for 48 h, and the percentage of apoptosis was detected by flow cytometry using Annexin V/propidium iodide (PI) staining. The combined treatment significantly increased the percentage of apoptosis in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation by 22% (synergistic) and 18% (sensitising) as compared to cisplatin alone (A). An increase in the percentage of apoptosis by 36% for the sensitising effect was noted in the double-positive ($CD166^+/EpCAM^+$) subpopulation of H2170 cells compared to cisplatin alone. No apparent changes were observed between treatments for the synergistic effect, suggesting that single treatments of curcumin and cisplatin alone induced a maximum response to the cells (B). $^*P<0.01$, $^{**}P<0.001$; t-test.

Curcumin enhances the sensitivity of the CSC subpopulation of $CD166^+/EpCAM^+$ cells to cisplatin-induced apoptosis. The apoptotic effects of the combined treatment of curcumin either by synergism with cisplatin or sensitising effects prior to treatment with low dose cisplatin in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation was examined using the apoptosis assay (Annexin V/PI) 48 h post-treatment. The unsorted A549 and H2170 cells were used as a control to indicate the basal level of apoptosis following the treatments (Fig. 6). As shown for the synergistic effect of curcumin (Fig. 6A); the results indicated that single treatments of curcumin and cisplatin induced apoptosis in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation of A549 cells to an average of 14 and 15% following 48 h of treatments. Moreover, the apoptotic effect was significantly increased to an average of ~37% in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation as these two treatments were applied simultaneously. The results also showed that curcumin sensitisation prior to treatment with low dose cisplatin in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation of A549 cells substantially increased the percentage of apoptosis by ~20% as compared to treatment with low dose cisplatin with only 2% apoptosis (Fig. 6A).

There were no significant changes in the percentage of apoptosis between the single treatment of curcumin or cisplatin, and the combination treatments by synergistic effects on sorted H2170 cells (Fig. 6B). This result suggests that the cells are highly sensitive to both curcumin and cisplatin; the IC_{50} concentrations of both treatments given to the cells have

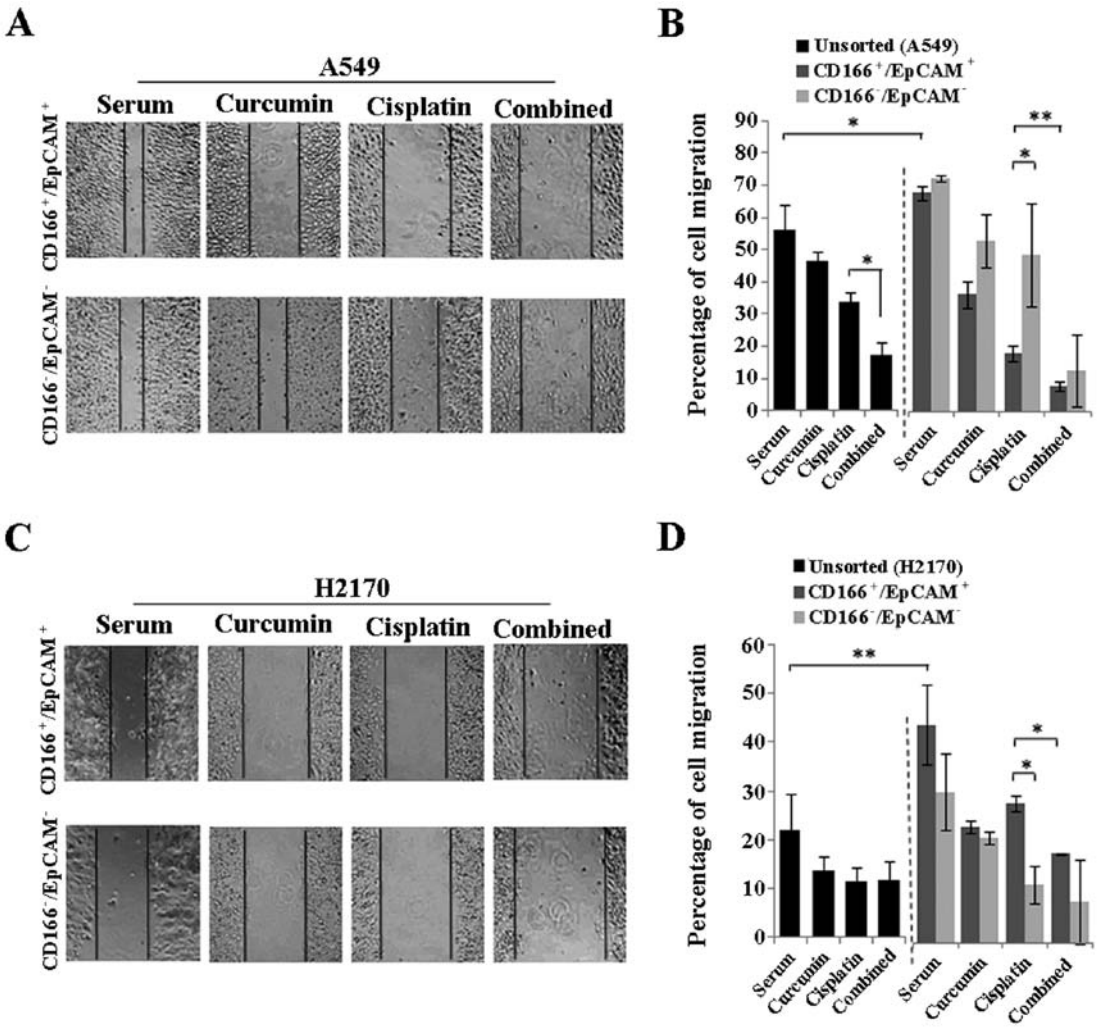

Figure 7. Curcumin enhances the cisplatin-induced metastatic inhibition of the CSC subpopulation ($CD166^+/EpCAM^+$) in the NSCLC cell lines. Representative photomicrographs and analysis of wound closure are presented for sorted NSCLC cell lines after 48 h of curcumin treatment either alone or in combination with cisplatin using the IC_{50} values (A and C). Higher migratory potential was noted in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation as compared to the unsorted of both NSCLC cell lines (B and D). Combination of both curcumin and cisplatin markedly inhibited the migration of CSCs in both the A549 and H2170 cells, suggesting better efficacies of the combination treatment for inhibiting the highly migratory CSC subpopulation (B and D). $^*P<0.01$, $^{**}P<0.001$; t-test.

already induced a maximal response. Interestingly, we noted that by sensitising the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation of H2170 cells to curcumin, prior to treatment with low dose cisplatin notably enhanced its apoptotic effect by 40%, compared to only 20% apoptosis as observed for the synergistic treatments. Combination treatments by sensitisation of the double-positive $CD166^+/EpCAM^+$ CSC subpopulation of H2170 cells to curcumin, also significantly increased the percentage of late apoptosis to 35%, compared to treatment with low dose cisplatin with only 4% detected (Fig. 6B).

Curcumin enhances the cisplatin-induced inhibition of the metastasis of the highly migratory CSC subpopulation ($CD166^+/EpCAM^+$) in the NSCLC cell lines. To evaluate the migratory potential of the CSC subpopulation in the NSCLC cell lines (A549 and H2170) and the effects of curcumin either alone or in combination with cisplatin to inhibit the migration of these cells; scratch wound (migration) assay was performed in the sorted ($CD166^+/EpCAM^+$ and $CD166^-/EpCAM^-$) and unsorted NSCLC cell lines 48 h post-treatment. As depicted in Fig. 7B and D, the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation had a significantly higher migratory potential as compared to the unsorted cells observed for both NSCLC cell lines. The combination of curcumin and cisplatin reduced the percentage of cell migration from 33.8 (cisplatin) to 17.3% (combined) in the unsorted A549 cells (Fig. 7B) with no apparent changes as noted in the H2170 cells (Fig. 7D). Furthermore, combined treatment markedly inhibited the migration of the $CD166^+/EpCAM^+$ subpopulation from 19.6 to 8.7% in the A549 cells and from 32.6 to 20.9% in the H2170 cells as compared to cisplatin treatment alone (Fig. 7B and D). Moreover, curcumin alone was able to inhibit the migration of the $CD166^+/EpCAM^+$ subpopulation in both the A549 and H2170 cells signifying the potential of curcumin on CSC inhibition. A higher cell migration was also noted in the $CD166^-/EpCAM^-$ compared to the $CD166^+/EpCAM^+$ subpopulation for the cisplatin treatment alone in A549 cells, while an opposite effect was noted in the H2170 cells (Fig. 7B and D). However, there were no differences in the percentage of cell migration between the $CD166^+/EpCAM^+$ and $CD166^-/EpCAM^-$

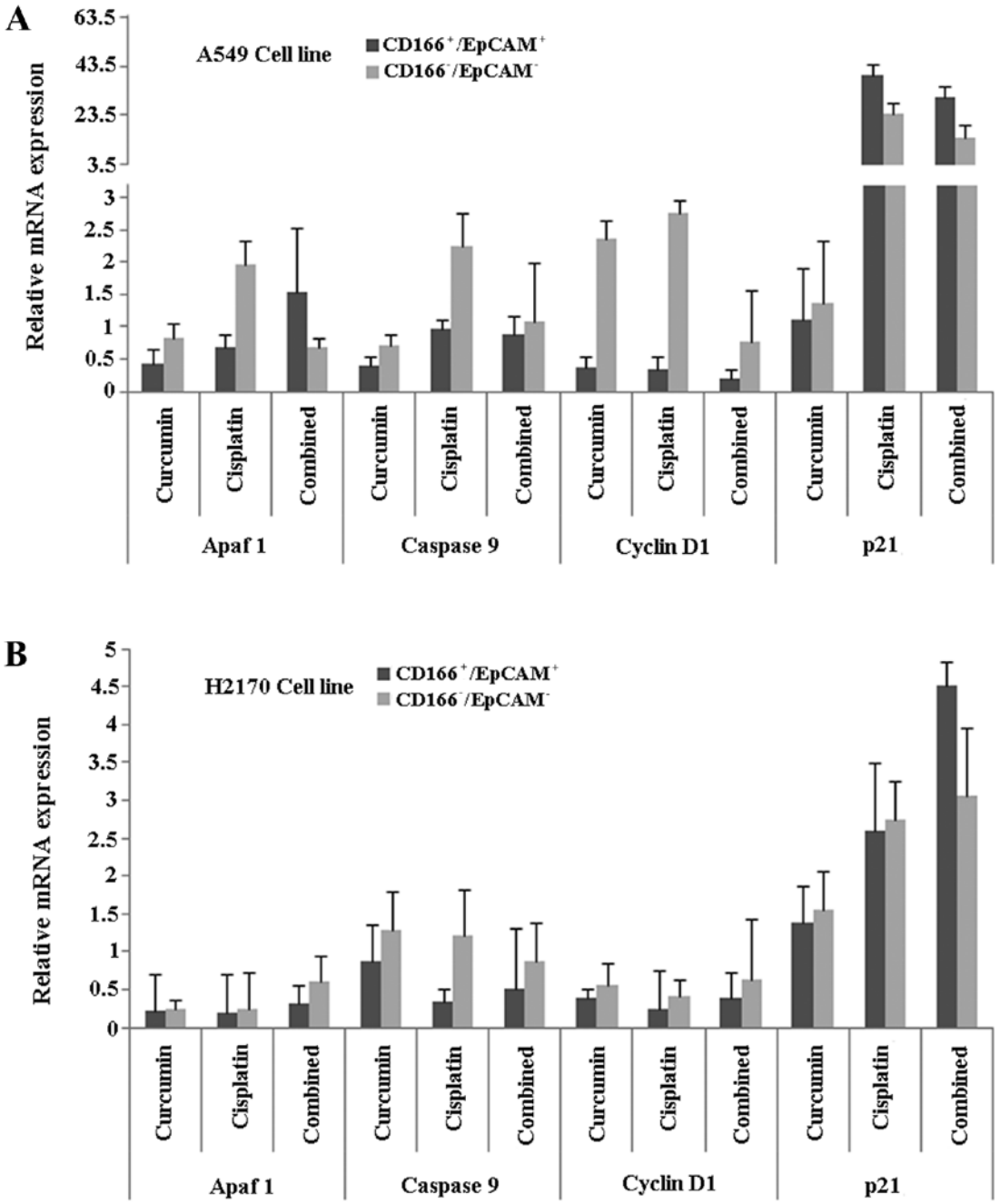

Figure 8. The mRNA expression of apoptotic (Apaf1 and caspase-9) and cell cycle-regulating (cyclin D1 and p21) genes, 48 h post-treatment. The mRNA expression of selected genes was evaluated in A549 (A) and H2170 (B) cells after treatment with the combination of curcumin and cisplatin by direct combination of both (synergistic effects) based on the IC_{50} values.

subpopulation for the combination treatment in both NSCLC cell lines (Fig. 7B and D).

Curcumin together with cisplatin increases the positive expression of apoptotic and cell cycle-regulating genes in the sorted cells. Finally, in order to understand the mechanisms behind the process, we investigated specific genes involved in apoptosis (Apaf1, cytochrome *c* and caspase-9) and cell cycle regulation (cyclin D1 and p21) in the double-positive ($CD166^+/EpCAM^+$) CSC subpopulation of both A549 and H2170 cells, after induction of treatments using either curcumin or cisplatin, and the combination of both. The results showed that the relative gene expression level of Apaf1 was higher in the combined treatment group compared to the single treatments (curcumin or cisplatin) in the $CD166^+/EpCAM^+$ subpopulation of A549 cells (Fig. 8A). Furthermore, the expression of p21 was high, with low expression of the cyclin D1 gene, in the $CD166^+/EpCAM^+$ subpopulation of both the A549 and H2170 cells, as compared to the $CD166^-/EpCAM^-$ subpopulation in the combined treatment group (Fig. 8A and B). Combined treatments induced high expression of caspase-9 in the $CD166^+/EpCAM^+$ subpopulation of A549, compared to single treatments of curcumin (Fig. 8A). On the other hand, the expression of caspase-9 was consistently low in the

$CD166^+/EpCAM^+$ subpopulation of H2170 cells for all of the treatments (Fig. 8B).

Discussion

The existence of chemoresistant tumour cells is one of the major obstacles reducing the efficacies of antitumour agents for cancer treatments. Studies have demonstrated that CSCs, as the main component in the tumour that drives tumour invasion, metastasis and relapse, are also believed to be the main reason for the chemoresistant phenotype. Currently, cisplatin and other platinum-based compounds are the most effective agents for the treatment of lung cancer patients, and they are usually combined with other agents such as docetaxel, gemcitabine and paclitaxel to yield higher efficacies (57). However the use of conventional drugs is limited due to the side effects and the resistant phenotype acquired by tumours (58,59). Active compounds derived from plants, microbes and marine organisms have been the interest of many investigators recently. These active compounds either in their crude or purified extracts have been shown to either have synergistic effects with chemotherapy or sensitising effects on CSCs, thus yielding superior efficacy as compared to chemotherapy alone (60,61). It is also suggested that the sensitising effects of these active compounds, might be useful to reduce the toxicity in patients by high dose chemotherapy.

In the present study, we studied the efficacy of curcumin, a natural compound extracted from *Curcuma longa*, either alone or in combination with cisplatin on the inhibition of double-positive ($CD166^+/EpCAM^+$) CSC subpopulation sorted from NSCLC cell lines (A549 and H2170), that we previously characterised (55). Curcumin cytotoxicity in NSCLC cell lines indicated by the IC_{50} values (Table II) showed that the agent, similar to cisplatin, is able to inhibit NSCLC cell proliferation. Moreover, sensitisation of both NSCLC cell lines using curcumin prior to treatment with low dose cisplatin, significantly reduced the percentage of viability in both the NSCLC cell lines compared to the treatment with low dose cisplatin (Fig. 2C and D). These results are in agreement with studies that have shown similar findings on the ability of curcumin to enhance the effects of cisplatin in NSCLC cell lines (23,62). These findings might also suggest that through the sensitising effects of curcumin, a combination of both curcumin and cisplatin could potentially be used as a treatment strategy to compliment the effects of low dose ciplatin. Thus, a higher therapeutic efficacy with lower toxicity can be achieved. Moreover, the IC_{50} value of curcumin, as well as cisplatin, that were evaluated in the NSCLC cell lines did not induce cytotoxicity on normal epithelial cells (IMR-90) (Fig. 2E) indicating that the target effects of both agents are tumour-specific.

The presence of CSCs as part of the tumour population has recently become the interest of many investigators. Most studies believe that by specifically targeting these subpopulations, the efficacy of treatments could be enhanced and eventually might reduce the chances for relapse (56). However, the major obstacles to this approach are the resistance characteristics of CSCs upon treatment (63). We noted that curcumin enhanced the induction of apoptosis by cisplatin in the $CD166^+/EpCAM^+$ subpopulation in both A549 and H2170 cells by either sensitising or synergistic effects (Fig. 6). Interestingly, in H2170 cells, synergistic effect of curcumin by direct combination with cisplatin did not induce significant changes in the percentage of apoptosis in the $CD166^+/EpCAM^+$ subpopulation of this cell line, as compared to cisplatin alone (Fig. 6B). However, the percentage of apoptosis was significantly increased when the subpopulation was initially sensitised to curcumin, prior to treatment with low dose cisplatin (Fig. 6B). Based on this observation, we believe that curcumin has the potential to alter the phenotype of NSCLC cells to treatments by enhancing the sensitivity of CSCs to chemotherapy. This theory is supported by few studies that have shown the same effects in several tumour models such as breast tumours and colon cancer, where these investigators have attributed the effects of curcumin on the inhibition of CSCs (64,65). These results also suggest that the approach of utilising curcumin either by direct combination (synergistic) or indirect combination (sensitising) with cisplatin should be taken into consideration if efficacy of the combined treatment is to be optimal on inhibiting the CSC subpopulation. Moreover, analysis on the gene expression level in the $CD166^+/EpCAM^+$ subpopulation of both A549 and H2170 cells from our previous study also demonstrated that there are variations in the tumourigenic mRNA expression between this subpopulation that we believed could further be attributed to the heterogeneity of CSCs to treatment outcomes (55).

The properties of CSCs, a subpopulation of cells that exhibit stem cell characteristics and contribute to treatment resistance, have been suggested as a candidate for mediating metastatic progression (66). In contrary, other cancer cells which do not exhibit stem cell characteristics and metastasise into distant tissue and confront an entirely new microenvironment may often be unable to colonise and grow. Only CSCs with high EMT (epithelial to mesenchymal transition) characteristics and a resistant phenotype have the capacity to metastasise and survive long enough and arrive at distant sites (67). Our results presented here showed that the $CD166^+/EpCAM^+$ subpopulation of both NSCLC cell lines have substantially higher migratory potential as compared to the unsorted cells (Fig. 7), consistent with the high metastatic characteristics of CSCs, that have been shown by several studies (68,69). Furthermore, a combination of both curcumin and cisplatin markedly inhibited the migration of the $CD166^+/EpCAM^+$ subpopulation in both A549 (Fig. 7A and B) and H2170 cells (Fig. 7C and D) compared to cisplatin treatment alone, indicating that the combination treatment induced superior effects on inhibiting the migration of CSCs. This finding might also indicate that the synergistic effects of both treatments could be utilised as a treatment strategy to combat the highly migratory CSC subpopulation. Thus, the probability of metastatic progression after chemotherapy in NSCLC may be reduced.

We observed that the combination treatment reduced the mRNA expression of cyclin D1 and induced p21 expression in the $CD166^+/EpCAM^+$ subpopulation in both the A549 and H2170 cells that eventually halted the growth of these cells (Fig. 8). These results are consistent with previous studies, demonstrating that curcumin by itself, has the potential to alter cyclin D1 and p21 expression and in combination with common chemotherapeutic drugs, the inhibitory effects were enhanced

through the inhibition of CSCs (70,71). Although we noted that the combination treatment enhanced mRNA expression of Apaf1 and caspase-9 in the CSC subpopulation of A549 cells, compared to cisplatin treatment alone, the combination treatment did not influence Apaf1 and caspase-9 expression in the CSC subpopulation of H2170 cells. We believed that this is due to the heterogeneity of CSCs that leads to the different sensitivity of the double-positive ($CD166^+/EpCAM^+$) subpopulation to the treatment outcome.

In conclusion, we showed that curcumin is able to increase the efficacy of low dose cisplatin in unsorted NSCLC cell lines. Through our investigation of the sorted NSCLC cell lines, we also found that curcumin had the capacity to enhance cisplatin-induced metastatic inhibition and apoptosis of the highly migratory CSC subpopulation ($CD166^+/EpCAM^+$) in the NSCLC cell lines suggesting that curcumin might be useful as a complement to common chemotherapy for inhibiting tumour progression and reducing metastasis.

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