DOWN-REGULATION OF ALDH1A1 INCREASED EXPRESSION OF CARCINOGENESIS-RELATED GENES IN NON-SMALL CELL LUNG CANCER CELL LINE OF A549

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Abstract

Purpose: This study was aimed to isolate the putative cancer stem cell (CSC) populations from A549 lung cancer cell line and to evaluate the difference of carcinogenesis-related genes expression within parental population (A549) and isolated CSC populations (A549 CD166+/EpCAM+ and CD166+/CD44+). Methods: We performed flow cytometry analysis to sort out cell positive for these markers; CD166+/EpCAM+ and CD166+/CD44+ from A549 cancer cell line. The isolated cells were tested for multipotent capacities by clonogenic and differentiation assays. Quantitative real time PCR was performed for both isolated CSC population and parental population to test expression of ALDH1A1 and 6 other genes that known to contribute to carcinogenesis; RARβ CYP24A1, BIRC5, EDN1, IL1β and PTGS2. Result(s): Both CD166+/EpCAM+ and CD166+/CD44+ have ability to form colonies and able to differentiate into adipocytes and osteocytes. Expression of ALDH1A1 was downregulated in all three cell populations (parental A549, A549 CD166+/EpCAM+ and A549 CD166+/CD44+) whereas the other 6 carcinogenesis-related genes were upregulated in all three cancer cell populations. There are no significant differences of gene expressions were detected among all three populations (p > 0.05). Conclusion(s): Downregulation of ALDH1A1 in all three cancer populations up-regulate the expression of other 6 carcinogenesis-related genes. Gene regulations between parental cancer cell (A549) and both putative CSC populations show no significant difference suggesting the existence of various CSC subpopulations reside within parental A549 population.

Keywords: Lung cancer, cancer stem cell, CD166+/EpCAM+ and CD166+/CD44+

Abstrak

Tujuan: Kajian ini adalah bertujuan untuk memencilkan populasi sel tunjang kanser (STK) daripada sel paru-paru A549 dan untuk menilai perbezaan ekspresi gen yang berkait rapat dengan pertumbuhan kanser dalam populasi induk (A549) dan populasi STK yang dipencilkan (A549 CD166+/EpCAM+ and CD166+/CD44+). Kaedah: Analisis flow-cytometry dijalankan untuk memencilkan sel yang positif pada penanda permukaan sel; CD166+/EpCAM+ and CD166+/CD44+ daripada sel induk A549. Sel yang dipencilkan diuji kebolehupayaan mampu membentuk koloni dan mempunyai kemampuan membeza menjadi adipoplast dan osteoplast. Tindak balas polimerase berantai kuantitatif dijalankan bagi kedua-dua populasi STK dan populasi induk bagi menguji ekspresi gen ALDH1A1 dan 6 gen lain yang dikenali menyumbang kepada pembentukan kanser; RARβ CYP24A1, BIRC5, EDN1, IL1β and PTGS2. Kesimpulan: Kedua-dua CD166+/EpCAM+ and CD166+/CD44+ subpopulasi mempunyai keupayaan membentuk koloni dan boleh membeza kepada...
1.0 INTRODUCTION

Lung carcinoma is the most common cause of cancer-related mortality worldwide. According to a report of Malaysia Cancer Statistics, a total of 18,219 new cancer cases were diagnosed among Malaysian population in 2007 [National Cancer Registry 2011]. Lung cancer is the third highest percentage of cancer that occurs in Malaysia after breast and colorectal cancer with the percentage of 10.2%. Among Malaysian male population, colon cancer was listed as the most common cancer with the percentage of 16.3% in 2006 [1]. Lung cancer is often diagnosed at an advanced stage and has a poor prognosis with overall survival rate is lower than 15% [2].

Lung cancer is often incurable and remains the leading cancer killer in both men and women [3]. There are two main categories of lung tumours; small cell lung cancer (SCLC) that represents 20% of all lung cancers and the most common form of lung tumour which is non-small cell lung cancer (NSCLC). NSCLC can be subdivided into three major histologic subtypes including adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC) [4]. The main challenge in cancer treatment is cancer recurrence and recent studies suggested that this is caused by a minority of cancer cells which exhibit similar characteristics as normal stem cells, named as cancer stem cells (CSCs) [5]. These rare subpopulations of undifferentiated cells have the unique biological properties necessary for tumour initiation, maintenance and spreading [5].

CSCs possess capacity similar to normal stem cell for proliferation, self-renewal and differentiation. CSCs that have capacity to initiate tumours have been linked to therapeutic resistance [6]. CSCs can be identified by three unique properties; (1) the expression of a distinctive set of surface biomarkers, which allows it to reproducible and differential purification (2) it is selectively endowed tumorigenic capacity as opposed to all other subsets (3) the ability to recreate repertoire of cancer cells of the parent tumour, thus displaying two of the functional characteristic of stem cells including self-renewal and differentiation [5].

The presences of CSCs have been identified by several methods, one of those based on the expression of specific surface markers within several solid malignancies. There are several specific surface markers for CSCs including EpCAM, CD166 and CD44. Epithelial cell adhesion molecule (EpCAM) is also known as CD326 [7]. EpCAM is a glycosylated, 30-40kDa type I transmembrane glycoprotein that functions as an epithelial-specific cell adhesion molecule [8, 9]. EpCAM is expressed in a variety of human epithelial tissues, cancers and progenitor and stem cells [9]. Similar with EpCAM, CD166 is also a cell adhesion molecule. CD166 which is also called as ALCAM (activated leukocyte cell adhesion molecule) associated with adenoma to carcinoma development [10]. CD166 has been observed to be upregulated in highly metastasizing melanoma cell lines, suggesting a role in tumour migration. Another CSCs surface marker is CD44. CD44 that involves in cell-to-cell and cell-to-matrix interactions [11] participates in many cellular processes including growth, survival, differentiation and motility [12]. These three surface markers had been reported to be strongly expressed in carcinomas of various origins including lung [13], colon and rectum [14], prostate [15], liver [16, 17], oesophagus [18], head and neck [19] and pancreas [20].

In this study, the aimed was to isolate the putative CSC populations from A549 lung cancer cell line using cell surface markers of CD166, EpCAM and CD44. The study of the expression pattern of genes related to carcinogenesis and their links with CSCs maintenance can be beneficial for targeting new cancer therapy. The gene expression study was performed as to evaluate the difference of carcinogenesis-related genes expression within parental population (A549) and isolated CSC populations (A549 CD166+/EpCAM+ and CD166+/CD44+). The genes of ALDH1A1 (participates in CSCs maintenance) [21], RARβ (regulator for retinoid signalling), CYP24A1 (reduce efficacy of antiproliferative; vitamin D3), BIRC5 (anti-apoptosis) [22], EDN1 (neoangiogenic and
mitogenic), IL1β (immunomodulator) and PTGS2 (mitogenic) were selected based on their roles in tumor development.

2.0 METHODOLOGY

2.1 Cell Culture

Human lung cancer cell line A549 was purchased from American Type Culture Collection (ATCC, Manassas, VA). A549 was cultured in RPMI-1640 [Invitrogen, Carlsbad, CA, USA] medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all purchased from ATCC). Cell was incubated at 37°C supplied with 5% CO₂. The culture was maintained by replacement of medium every two to three days. Confluent culture was washed with phosphate buffer saline (PBS) and was trypsinized by 0.25% trypsin in EDTA for subculturing. All reagents were purchased from Gibco-Life technologies (Grand Island, NY, USA).

2.2 Isolation Of Cell Subpopulation

Cells positive for CD166, CD44 and EpCAM were sorted out. The A549 cell line was harvested using 0.25% trypsin- EDTA and washed with 2% FBS. The percentages of putative CSCs were accessed by measurement the fluorescent intensity of cell surface markers using flow cytometer. Cells were labelled with PE-conjugated anti-CD166 (Clone: 3A4; Isotype: Mouse Ig1, κ), FITC- conjugated anti-CD44 (Clone: L178; Isotype: Mouse IgG1, κ) and FITC-conjugated anti-EpCAM (Clone: 158206; Isotype: Mouse IgG2B; Isotype: Mouse IgG1, κ) (R&D System, Minneapolis, MN, USA) as described by [23]. Labelled cell was analyzed and sorted with a mo-flow cytometer to obtain CD166+ CD44+ and CD166+ EpCAM+ subpopulations. The sorted lung cancer stem cells then were cultured and expanded in vitro until confluent.

2.3 Stemness Evaluation

The capabilities of sorted CSCs to differentiate into different lineages were determined by performing clonogenic and differentiation assays.

2.3.1 Clonogenic Assay

Survival of the sorted CSCs was defined as the ability of the cells to maintain their clonogenic capacity and form colonies. CD166+ CD44+ and CD166+ EpCAM+ subpopulation cells were trypsinized and counted. 1 × 10⁴ cells were seeded for colony formation in the T25 culture flask. After 14 days incubation, the colonies were stained with crystal violet and manually counted. Colonies containing more than 50 cells were scored as described in Zhang et al., (2004)[24].

2.3.2 Osteogenic Assay

1 × 10⁴ cells were seeded in 24-well plates and grown 72 hours until reach 100% confluency. Cells were cultured for 2 weeks in osteogenic medium (StemPro, Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37°C supplied with 5% CO₂. The culture was maintained by replacement of osteogenic medium every two to three days. Extent osteogenic differentiations were determined by deposition of calcium. Calcium deposits were visualised by staining with Alizarin Red S.

2.3.3 Adipogenic Assay

1 × 10⁴ cells were seeded in 24-well plates and grown 72 hours until reach 100% confluency. Cells were cultured for 2 weeks in adipogenic medium (StemPro, Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37°C supplied with 5% CO₂. Culture was maintained by replacement of adipogenic medium every two to three days. To visualize adipocytes, cultures were fixed with paraformaldehyde and stained with Oil Red.

2.4 Isolation Of Total RNA

Total RNA from approximately 2 × 10⁶ cells of lung CSCs were isolated using Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instruction. Briefly, the cells were lysed with lysis buffer and homogenised using the QIAahredder Homogenizer (Qiagen). The homogenised cell lysates were added with 70% ethanol and then were transferred into the RN spin column. Total RNA was eluted from spin column using RNasefree water. The concentration and purity of extracted RNA were determined using a Nanodrop ND1000 spectrophotometer, and the RNA integrity number (RIN) was determined using the Bioanalyzer 2100 (Aglent Technologies).

2.5 Real-Time PCR (qRT-PCR)

cDNA were synthesized from total extracted RNA by using Maxima First strand CDNA synthesis kit (Thermo Fisher Scientific, USA). In order to determine the expression of ALDH1A1 gene in isolated CSCs, real-time PCR (qRT-PCR) was performed. qRT-PCR was performed for both A549 parental and A549 sorted cells (CD166+ CD44+ and CD166+ EpCAM+) by using QuantilNovo SYBR Green PCR Kit (Qiagen, Germany). ALDH1A1- related genes; BIRC5, RARβ, CYP24A1, IL1B, EDN1 and PTGS2 expression level were accessed in the same manner. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was amplified as reference standard. In order to compare the expression data between parental A549 with isolated CSCs, a T-test were carried out with p<0.05 was considered statistically significant.
3.0 RESULTS AND DISCUSSION

3.1 Isolation Of CD166+/Epcam* And CD166+/CD44* Subpopulations From A549 Cell Lines

Ability of CSCs in promoting and maintaining cancer cell population even after surgery and chemotherapy suggesting that targeting CSCs might be the best target for new cancer therapies. Finding the specific markers for identification of putative CSCs is the most critical issue [25]. In the previous study, our group found that CD166 was highly expressed in A549 cell line [26]. Thus, identifying the stringent phenotypes of CSCs, we investigated co-expression of CD166 marker with EpCAM and CD44 markers.

The expression of CSC subpopulations; CD166/EpCAM and CD166/CD44 in A549 cell lines were studied. Co-expression of two markers was investigated in order to obtain more stringent phenotypes of putative CSCs population. Small population of cells showed positivity for co-expression of CD166/EpCAM (2.11%) whereas approximately half population of cells showed positivity for co-expression of CD166/CD44 (49.32%) (Fig.1). The double positive cell for CD166/EpCAM and CD166/CD44 were isolated and expanded for further downstream experiment.

The flow cytometry analysis demonstrated that the NSCLC of A549 cell line not only consists of CD166/EpCAM* and CD166/CD44*, but also other population of cells including CD166*/EpCAM*, CD166*/EpCAM, CD166*/CD44, CD166*/CD44* and CD166*/CD44. These results support the initial hypothesis that CSC population is heterogeneous.

Table 1: Human primer sequences used for qRT-PCR

<table>
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<th>Gene</th>
<th>Accession</th>
<th>Sense primer (5' – 3')</th>
<th>Antisense primer (5' – 3')</th>
<th>Product size (bp)</th>
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<td>AGGATAGGACTTGGGGGTCA</td>
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<td>RARB</td>
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<td>PTGS2</td>
<td>NM_000963.3</td>
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<tr>
<td>CYP24A1</td>
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<tr>
<td>EDN1</td>
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<td>TAGCCAAATGTTGTACATACC</td>
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</table>
3.2 Multipotent Characteristics Of Isolated Cscs

The multipotent characteristics of putative CSCs were accessed by their ability to self-renew and differentiate into multilineage cells. In this study, we hypothesized that double positive cell populations; CD166+/EpCAM+ and CD166+/CD44+ are the putative CSCs in A549 cell lines. This hypothesis was proven when both isolated CD166+/EpCAM+ and CD166+/CD44+ showed multipotent capabilities of cancer stem cells; ability to self-renew and differentiate into adipocytes and osteocytes lineages (Fig. 2) [27].

3.3 Gene Expression Of Carcinogenesis-Related Genes

ALDH gene superfamily encodes enzymes that play crucial role in certain processes in maintaining life via formation of molecules such as retinoic acid, betaine and gamma-aminobutyric acid and detoxification via NAD(P)⁺-dependent oxidation of endogenous and exogenous aldehydes [28-30]. The latest database showed that in human genome, ALDH gene superfamily contains 19 known putatively functional genes [30]. ALDH1A1 which is one of the ALDH superfamilies are cystosolic and are involved in acetaldehyde metabolism, retinal oxidation and detoxification of cyclophosphamide [30]. Previous study on ALDH1A1 proved that this isoform of ALDH is a CSC marker and are expressed highly in hematopoietic stem cells [31], lung cancer [32] and any other solid tumours and are associated with worse clinical outcome [33]. The importance of this enzyme in cell homeostasis might suggest that this enzyme can confer potentially great impact on lung cancer treatment [28].

Our gene expression study reveals that ALDH1A1 that encodes for ALDH1A1 enzyme which participates in the maintenance of CSCs was shown to be down-regulated in parental cancer cell and both putative CSC population cells (CD166+/EpCAM+ and
CD166+/CD44+). Consistent with our result, a study reported by Okudela and colleagues found that ALDH1A1 expression was markedly reduced in 39.9% out of 268 NSCLC patients that were tested in Japan [21]. The down-regulation of ALDH1A1 was led to up-regulation of other 6 carcinogenesis-related genes suggesting that this gene might play regulatory function in lung cancer (Fig. 3). Thus, in order to prove the importance of ALDH1A1 function in NSCLC, modification of ALDH1A1 should be performed in the future.

T-test analysis was performed to evaluate the difference of carcinogenesis-related genes expression within parental population (A549) and isolated CSC populations (A549 CD166+/EpCAM+ and CD166+/CD44+). However, the result showed that there was no significant difference in gene expression between parental A549 and both putative CSCs. This result suggesting that the parental A549 comprises of a heterogenous population of cancer cell (p>0.05) [26].

Figure 3 Expression of ALDH1A1 and carcinogenesis-related genes in A549 parental cell A549 CD166+/EpCAM+ and A549 CD166+/CD44+.

4.0 CONCLUSION

The putative CSC populations were successfully isolated from A549 lung cancer cell line using CD166+/EpCAM+ and CD166+/CD44+ markers, and it was demonstrated that the cells possess the stem-cell like characteristics; self-renewal capability and ability to differentiate into adipocytes and osteocytes lineages. Down-regulation of ALDH1A1 that led to up-regulation of other six carcinogenesis-related genes suggested that this gene might play an important role in promoting carcinogenesis in NSCLC. The modification of this gene for stem cell-based gene therapy might be able to down-regulate the other six carcinogenesis-related genes, thus disrupt the stemness capability of CSCs.

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