Cancer stem cell isolation and characterization from lung cancer cells

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Abstract

Lung cancer is one of the most common cancers in the world and a leading cause of death. Cancer stem cells (CSCs) are the cells responsible for tumor initiation. CSCs features include self-renewal capacity, differentiation, high invasion-migration, and resistance to chemotherapy. CSCs are characterized by specific surface markers. CD133 is widely used as a marker of CSCs in many cancers, including lung cancer. CD133-positive cells are an important marker of lung cancer stem cells because they show characteristics of lung cancer CSCs. CSCs are less sensitive and/or resistant to conventional treatments, remaining viable and regenerating tumors after treatment. Treatments that can target CSCs will be able to destroy CSCs more effectively, leading to the disappearance of tumors. Currently, attempts are being made to develop therapeutics that can effectively target CSCs. These studies concern the isolation and characterization of CSCs. In this study, CD133-positive CSCs and CD133-negative cells were isolated from H460 cells, a large cell lung cancer model, by MACS method. CSCs properties were investigated by tumorSphere formation assay (2% agar plate), hanging drop assay and qPCR method. CD133 positivity of H460 cells was determined as 0.3%. It has been observed that CD133-positive cells show CSCs-specific self-renewal properties. It was determined that the CD133 gene expression difference between CD133-positive CSCs and CD133-negative cells was approximately 25-fold. It has been observed that CD133-positive CSCs express more pluripotency genes (such as OCT-4, SOX-2 and KLF-4) compared to CD133-negative cells.
Introduction

Lung cancer is among the most common and deadly cancers worldwide (Sung et al., 2021). The use of tobacco and tobacco products are the main risk factors for developing lung cancer (Parkin et al., 1994). Non-small cell lung cancer (NSCLC) is the most prevalent type of lung cancer. The subtypes of the NSCLC include large cell carcinoma, adenocarcinoma, and squamous cell carcinoma (Howlader, 2011).

Treatment of lung cancer varies according to the stage of the disease. The treatment method utilized at the early stage is usually surgery and if necessary, adjuvant chemotherapy. For third-stage patients, chemotherapy and radiotherapy are usually administered concurrently. Treatment methods vary according to the subtype of the disease, the mutations involved, tumor location, metastases, and the age of the patient (Gadgeel et al., 2012).

In 1997, Bonnet and Dick showed that CD34 (+), CD38 (-) cells in acute myeloid leukemia differentiate into leukemic cells in NOD/SCID mice and self-renew. These stem-like leukemic cells were defined as cancer stem cells (CSCs) (Bonnet and Dick 1997). CSCs are a rare population of tumorigenic cells capable of tumor initiation, and maintenance (Pardal et al., 2003). These cells have unlimited proliferation potential, self-renewal ability, and the capacity to produce differentiated cells that make up the main tumor population (Eramo et al., 2008).

The properties of CSCs are thought to be regulated by stem cell transcription factors and multiple sets of molecular signals. Well-defined transcription factors used for CSC identification include Octamer-binding transcription factor 4 (OCT-4), Kruppel-Like Factor 4 (KLF-4), and Sex determining region Y-box 2 (SOX-2) (Maiuthed et al., 2018). These transcription factors share many target genes in embryonic stem cells. Direct interactions between SOX-2, KLF-4, and OCT-4 have been shown to be crucial for somatic cell reprogramming (Wei et al., 2009). Abnormal SOX-2 mRNA expression frequently results in increased treatment resistance and asymmetric cell division (Takeda et al., 2018). Elevated OCT-4 levels have been detected in aggressive tumors, relapse, and patients with poor prognoses. OCT-4 overexpression has been associated with tumorigenesis, metastasis, and cancer recurrence (Hayashi et al., 2015). KLF-4 acts as both a transcriptional activator and a repressor according to the cell type (Evans et al., 2007).

Human Cluster of Differentiation (CD) 133, is a plasma membrane protein and consists of transmembrane segments and large glycosylated extracellular domains (Weigmann et al., 1997). Studies show that CD133 (+) CSCs in lung cancer are more tumorigenic and drug-resistant (Bertolini et al., 2009; Eramo et al., 2008; Salnikov et al., 2010).

Therapeutics that aim to target CSCs are currently under development. Because most cancer models exist in a heterogeneous hierarchical model, drug, chemotherapy, and radiotherapy resistance develops in treatments applied only to cancer cells. However, it is possible to develop effective treatments by targeting the signaling pathways required for the maintenance of CSC characteristics (Pardal et al., 2003; Reya et al., 2001).

Magnetic-activated cell sorting (MACS) is one of the most utilized cell sorting methods, allowing the separation of cells using antibodies specific to cell surface antigens. MACS separation is based on the conjugation of antibodies to microbeads containing iron oxide. Following the binding of the conjugated antibodies to cells, a magnetic area is used to separate the labeled cells from the unlabeled cells (Tomlinson et al., 2013).

The core of cancer stem cell studies is the successful isolation of CSCs and the characterization of their cell-specific characteristics. In this study, cancer stem cell isolation and characterization were carried out from H460 cells, a large-cell lung cancer model, using the MACS method targeting the CD133 surface marker.
Cancer stem cell characteristics of CD133-positive CSCs and CD133-negative cells were investigated by tumorsphere formation assay (2% agar plate), hanging drop assay, and through analyzing mRNA expression levels of pluripotency associated-genes such as SOX-2, OCT-4, and KLF-4.

**Material and methods**

**Cell culture maintenance**

H460 cell line (Lot: 70015967) was used for cancer stem cell isolation and characterization. Adherent H460 cells were obtained from the pleural fluid of a male patient with large-cell lung carcinoma (ATCC, HTB-177). 10% FBS (Fetal Bovine Serum-Biological Industries, Israel) and 1% PS (Penicillin/Streptomycin-Biological Industries, Israel) (10,000 U/ml) were added into RPMI 1640 (Biological Industries, Israel) medium containing L-glutamine and phenol red. Cells were cultured with this medium according to standard mammalian tissue culture protocols (5% CO₂, 37 °C) according to ATCC guidelines using sterile techniques. After reaching 70% confluence, cells were harvested by trypsinization (0.25% Trypsin-EDTA solution, Biological Industries, Israel), counted using a Neubauer counting chamber (Isolab, Turkey), and used in the experiments described below.

**Cancer stem cell isolation**

CD133-positive CSCs and CD133-negative cells were isolated from H460 cells using the CD133 surface marker. CD133 MicroBead Kit from Miltenyi Biotec (Miltenyi Biotec, Germany) was used for MACS analysis. 7x10⁷ cells were used in the experiments.

The CD133-positive cells were magnetically labeled with CD133 MicroBeads. Then, the MACS column was put in the magnetic area of the MACS Separator, and the cell suspension was then loaded onto a MACS column. While magnetically labeled CD133-positive cells remained in the column, unlabeled CD133-negative cells passed down the column. After the column was removed from the separator, CD133-positive cells were dissociated.

**Cancer stem cell characterization**

Cancer stem cell characteristics of isolated CD133-positive CSCs and CD133-negative cells were examined by tumorsphere formation assay (2% agar plate), hanging drop assay, and qPCR method. Tumor spheres are floating spherical CSC models commonly used in CSC studies. To obtain spheres, CSCs are cultivated in serum-free medium enriched with factors such as epidermal growth factor (EGF), fibroblast growth factor (bFGF), insulin, and hydrocortisone (Breslin and O’Driscoll, 2013).

**Tumorsphere formation assay**

Sphere formation is specific to CSCs and demonstrates the self-renewal properties of CSCs; thus, this method was used to analyze the stem cell features. CD133-positive CSCs and CD133-negative cells were prepared as 500, 1000, 2000, 4000, and 6000 cells/well. CD133-positive CSCs and CD133-negative cells were grown in 2% agarose-coated 96 well plates (Tpp, Switzerland) using freshly prepared cancer stem cell medium (RPMI 1640 medium supplemented with growth factors, serum-free). For the preparation of cancer stem cell medium, 10 μL EGF (100 ng/mL) (Abcam, ab179628, USA), 10 μL FGF2 (20 ng/mL) (Abcam, ab9596, USA), 1 mL B27 supplement (Gibco, 17504-044, USA), 500 μL N2 supplement (Gibco, 17502-048, USA), and 500 μL Penicillin-Streptomycin (1%) (10,000 U/mL) (Biological Industries, Israel) were added into 50 mL RPMI 1640 medium (Biological Industries, Israel). To prepare the agar plate, 2 g of agarose (Multicel) was mixed with 100 mL of dH₂O and heated in a microwave oven for 2 minutes. The agar, which
became transparent by melting, was quickly placed on a 96-well plate. The agar was allowed to dry in the cabinet for 20 minutes and the dried agar plates were wrapped in aluminum foil and stored at 4 °C. Cells with cancer stem cell characteristics were expected to form floating spheres (Izumiya et al., 2012). Sphere formation was observed for 9 days and cell images were recorded with a Nikon, Eclipse Ts2 microscope (Manufacturer, Country).

**Hanging drop assay**

CD133-positive CSCs and CD133-negative cells were inoculated on the lid of a 100 mm petri dish and grouped into 250, 500, 1000, 2000, 4000, and 6000 cells in 20 μL of cancer stem cell medium. 10 mL of 1xPBS (Biological Industries, Israel) was added to the bottom of the petri dish. Cells were cultured at 37 °C and 5% CO₂ conditions, and spheroid formation was observed. Cell images were recorded with a Nikon, Eclipse Ts2 microscope (Raghavan et al., 2016).

**mRNA expression profile of transcription factors regulating CSCs**

The expression profile of OCT-4, CD133, SOX-2, and KLF-4 genes specific to CSCs were examined using qPCR. β-actin (ACTB) was used as a housekeeping gene. Primers specific for the KLF-4, OCT-4, ACTB, CD133, and SOX-2 genes were designed using the Primer-BLAST-NCBI, Ensembl, Primer3, and SnapGene (Table 1).

**Total RNA isolation from CD133-positive CSCs and CD133-negative cells**

500 μL of TRIzol (Invitrogen, USA) was added to CD133-positive CSCs and CD133-negative cells, and the cells were thawed. 100 μL of Chloroform (Sigma, Germany) was added and they were gently shaken until a color change was observed. Isolation was performed according to the manufacturer's guideline. 40 μL of DEPC water (Bio Basic, Canada) was added to the obtained RNA and the pellet was dissolved by pipetting. RNA samples were kept on ice and immediately measured for concentration and purity with NanoDrop (Thermo-Scientific, USA).

**cDNA synthesis**

SensiFAST cDNA Synthesis Kit (Meridian Bioscience, Germany) was used for cDNA synthesis. The kit products were thawed, vortexed, and spun down. for the cDNA synthesis reaction for each sample, 1 μg of total RNA was combined with 1 μL of Reverse Transcriptase, 4 μL of 5x TransAmp Buffer, and DNase/RNase-free water to have a total volume of 20 μL. The sample was mixed until it became homogeneous and incubated using Bio-Rad T100 device (Bio-Rad, USA) at 25 °C for 10 minutes, at 42 °C for 15 minutes, and at 85 °C for 5 minutes. After incubation, the tubes were immediately iced for 5 minutes. The obtained cDNAs were stored at -20 °C.

**Table 1. Primer sequences and PCR product lengths of SOX-2, OCT-4, KLF-4, CD133 and ACTB genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX-2</td>
<td>TTTGTCCGAGACGAGAAGGC</td>
<td>TAACTGTCCATGCGCTGGTT</td>
<td>237 bp</td>
</tr>
<tr>
<td>OCT-4</td>
<td>CAGTGCCCGAAAACCCACAC</td>
<td>GGAGACCCACGAGCCCTCAA</td>
<td>161 bp</td>
</tr>
<tr>
<td>KLF-4</td>
<td>AAGCCAAGAGGGGAAGACG</td>
<td>ATGTGAAGGGAGGGTGTC</td>
<td>287 bp</td>
</tr>
<tr>
<td>CD133</td>
<td>TCACCGAGTGAAACCCCGGA</td>
<td>TCGGTCTAGAACTCCGCTCT</td>
<td>193 bp</td>
</tr>
<tr>
<td>ACTB</td>
<td>TTCCTGGGCGATGGAGTCTCT</td>
<td>AGAGAGAGCAATGATCTTGATC</td>
<td>204 bp</td>
</tr>
</tbody>
</table>
Gradient PCR

Gradient PCR was first performed to define the binding temperature of the primers to be used in qPCR. A mixture of 16 µL 2x Master Mix (Biological Industries, Israel), 1 µL Forward Primer (10 pmol), and 1 µL Reverse Primer (10 pmol), and 2 µL of cDNA was prepared. Amplification was performed using the Bio-Rad T100 device (Bio-Rad, USA). After PCR, the correct amplification of SOX-2, CD133, OCT-4, ACTB, and KLF-4 genes was checked by loading the PCR products on 2% agarose gel. The gradient PCR conditions were 95 °C for 5 minutes (initial denaturation), 36 cycles of 95 °C for 30 seconds (denaturation), 58-64 °C for 30 seconds (annealing); 72 °C for 30 seconds (extension), and 72 °C for 5 minutes (final extension).

qPCR

mRNA expression levels of SOX-2, OCT-4, KLF-4, ACTB, and CD133 genes were determined quantitatively. SensiFAST SYBR Kit (Meridian Bioscience, Germany) was used for qPCR experiments. The reaction mixture was prepared to contain 0.8 µL Forward Primer (10 pmol), 0.8 µL Reverse Primer (10 pmol), 10 µL of 2x SensiFAST SYBR Mix, 3 µL cDNA and 5.4 µL DNase/RNase Free Water. A Bio-Rad CFX96 Real-time PCR device (Bio-Rad, USA) was used for amplification. The qPCR conditions were 95 °C for 2 minutes (initial denaturation) and 40 cycles of 95 °C for 5 seconds (denaturation); 60 °C for 10 seconds (annealing); and 72 °C for 20 seconds (extension).

Statistical analysis

The 2-ΔΔCT method was used to compare gene expressions of CD133 positive CSCs and CD133 negative cells. ΔΔCT values were calculated according to the following formula: ΔΔCt = (Ct target gene - Ct β-actin) CD133-positive cells - (Ct target gene - Ct β-actin) CD133-negative cells. The significance of group differences was assessed with Student’s two-tailed t-test using with SPSS v.28 (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

The most common cancer-related deaths in the world are lung cancer-related ones (Jemal et al., 2011). Most of the patients experience distant relapse because the current treatment methods are not sufficient. This is caused by micrometastatic spread of chemoresistant cells that survive (neo)adjuvant therapy. CSCs have been detected in various tumor types in recent years. These cells are predominantly characterized by the expression of CD133 (Singh et al., 2003; Ricci-Vitiani et al., 2007; Curley et al., 2009).

In our study, we separated lung CSCs expressing CD133 from H460 cells by MACS method, which revealed that the CD133 positivity of cells was 0.3%. It was determined that the CD133 mRNA expression difference between CD133-positive CSCs and CD133-negative cells was approximately 25-fold, and the MACS method was confirmed (Figure 1). Annealing temperatures were determined as 60 °C according to gradient PCR results (Figure 2).
3D micro-environments are known to be more suitable than traditional 2D systems for simulating in vivo conditions (Smith et al., 2011; Altmann et al., 2011). We used two well-known 3D culture methods in our study. The 3D growth potentials of CD133-positive CSCs and CD133-negative cells were analyzed by tumorsphere formation assay (plate covered with 2% agar) and hanging drop assay approaches. CD133-positive CSCs showed a CSCs-specific self-renewal feature. It was observed that CD133-positive CSCs formed spheroids on the 3rd day on the plates covered with 2% agar, while CD133-negative cells did not form spheroids. Sphere formations were followed for 9 days (Figure 3). With hanging drop cell culture, CD133-positive CSCs started to form spheroids on day 2, while CD133-negative cells did not form spheroids (Figure 4).

It has been shown in the literature that CD133-positive tumor spheres exhibit self-renewal under non-adherent conditions supported by growth factors. Eramo et al. showed that CD133-positive cancer stem-like cells, which are found in low amounts (<1%) in lung cancer, can self-renew and have high tumorigenic potential. They also confirmed the high tumorigenic potential of lung cancer spheres through in vivo studies. As these spheres regenerate the original lung tumor, they were suggested to provide an excellent model for studying lung cancer biology and response to therapeutic approaches (Eramo et al., 2008). CD133-positive cells isolated from tumor samples of patients with NSCLC have been shown to have a higher self-renewal capacity compared to CD133-negative cells. It has been confirmed that CD133-positive spheres grown in non-adherent media have higher tumorigenicity in NOD/SCID mice (Tirino, 2009). Wang et al. isolated CD133 (+) and CD133 (-) cells from A549 and H460 cells using CD133 MicroBeads (Miltenyi Biotec). They found CD133 positivity in A549 and H460 cells as 2.8% and 2.4%, respectively, by MACS method and observed that CD133 (+) CSCs form spheres (Wang et al., 2021).
Figure 3. Tumorsphere formation assay results of CD133-positive CSCs and CD133-negative cells. CD133-positive CSCs: A. 500 cells/well B. 1000 cells/well formed spheres. C. 1000 cells/well of CD133-negative cells did not form spheres. Magnification is x40.

Figure 4. Hanging drop assay results of CD133-positive CSCs and CD133-negative cells. A. and B. CD133-positive CSCs formed spheres. C. CD133-negative cells did not form spheres. Magnification is x40.
Arthur et al. isolated CD133 (+) stem cells using the MACS method and found the CD133 positivity of H460 cells to be 0.9-1% (Arthur et al., 2020). The pluripotency potential of CD133-positive CSCs and CD133-negative cells was examined by analyzing their OCT-4, KLF-4, and SOX-2 mRNA expressions. A 6-fold difference in OCT-4, 3-fold difference in KLF-4, and 2-fold difference in SOX-2 were found between the expression levels of CD133-positive and CD133-negative cells (Figure 5).

It is known that cancer stem cell properties are closely regulated by stem cell transcription factors that play a role in maintaining normal stem cell activities. Commonly used stem cell transcription factors used for cancer stem cell characterization include OCT-4, SOX-2, KLF-4, and Nanog (Maiuthed et al., 2018). The formation of in vitro tumor spheroids is also associated with increased levels of CD44, CD133, OCT-3/4, SOX2, and Nanog (Jin et al., 2017). OCT-4, also known as one of the Yamanaka Factors, is a member of the POU (Pit-Oct-Unc) transcription factor family (Schöler et al., 1990a) and is highly expressed in embryonic stem cells (Schöler et al., 1990b). Therefore, OCT-4 is a main promoter of stem cell pluripotency and differentiation (Pesce and Schöler, 2001). Lung cancer CD133 (+) cells have been shown to exhibit higher OCT-4 expression, increased invasion and self-renewal, resistance to therapy, and increased cancer stem cell abilities compared to CD133 (-) cells.

This finding demonstrated that OCT-4 plays an important role in maintaining cancer stem cell-like characteristics and chemo- and radiotherapy-resistance in CD133 (+) cells (Chen et al., 2008). CD133-expressing lung cancer cells exhibit elevated OCT-4 expression levels, which induce them to regenerate and metastasize. Recent studies have shown that the loss of OCT-4 gene expression causes CD133 (+) cells to lose their sphere-forming ability and induce them to differentiate back into CD133 (-) cells in human lung cancer (Prabavathy et al., 2018). SOX-2 gene is a member of the SOX family transcription factors and was identified in humans in 1994 (Stevanovic et al., 1994). Overexpression of SOX-2 is related to anchorage-independent growth, migration, and chemoresistance (Weina and Utikal, 2014). High levels of SOX-2 are required to maintain the self-renewal ability of lung cancer stem cells (Singh et al., 2012). Another well-known Yamanaka factor, KLF-4, can function as a tumor suppressor, oncogene transcriptional activator, and repressor (Rowland et al., 2005; Rowland and Peeper, 2006; Ghaleb and Yang, 2017). KLF-4 was found to be upregulated in a population of cells in lung cancer (Salcido et al., 2010). Significant increases in stemness genes (OCT-4, SOX-2, KLF-4 and C-MYC), and CSC-specific markers (CD133 and ALDH1) were observed in NSCLC patients (MacDonagh et al., 2017). KLF-4 has also been reported in recent studies to be a promoter of CSCs in various cancers (Leng et al., 2020; Qi et al., 2019; Zhu et al., 2014).

Figure 5. KLF-4, OCT-4 and SOX-2 mRNA expression levels in CD133-positive CSCs and CD133-negative cells. Asterisks indicate statistically significant differences (p<0.05).
Current treatment methods may fail to eradicate the population of CSCs, which is the primary cause of relapse. CSCs are capable of self-renewal, resistance to therapy, differentiation, invasion, and metastasis. Due to these features, they have become the new targets in cancer treatment approaches in recent years.

According to our study, CD133 positivity was found to be low as in the literature. Cancer stem cell properties were accurately characterized by tumorsphere formation assay (2% agar plate), hanging drop assay and qPCR method. In cancer stem cell research, the isolation and characterization of CSCs are fundamental stages. Proper execution of these steps will contribute to the acceleration of CSC studies.

**Conclusions**

CSCs are a rare group of cells that account for less than 1% of the cancer cell population in most solid tumors. As a result of our study, CD133 positivity was found to be 0.3% and the CSCs properties of these cells were characterized. The self-renewal properties of these cells have been proven by tumorsphere assays. According to the literature, the expression of pluripotency genes such as \( OCT-4 \), \( KLF-4 \), and \( SOX-2 \) in CD133-positive CSCs was expected to be higher than those of CD133-negative cancer cells. Compared to CD133-negative cells, CD133-positive CSCs were observed to express \( OCT-4 \), \( SOX-2 \), and \( KLF-4 \) genes, 6, 2, and 3 times more, respectively. These results show that CSCs were correctly isolated and characterized.

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**Conflict of interest**

Authors declare no conflict of interest.

**References**


