The role of CD133 in the identification and characterisation of tumour-initiating cells in non-small-cell lung cancer☆,☆☆

Virginia Tirino [a], Rosa Camerlingo [a], Renato Franco [b], Donatella Malanga [c,d],
Antonello La Rocca [e], Giuseppe Viglietto [c,d], Gaetano Rocco [e], Giuseppe Pirozzi [a,*]

[a] Department of Experimental Oncology, National Cancer Institute, Naples, Italy
[b] Department of Pathology, National Cancer Institute, Naples, Italy
[c] Department of Clinical and Experimental Medicine, University of Catanzaro, Italy
[d] Institute for Genetic Research "G Salvatore" Ariano Irpino (AV), Italy
[e] Department of Thoracic Surgery and Oncology, National Cancer Institute, Naples, Italy

Abstract

Objective: Emerging evidence suggests that specific sub-populations of cancer cells with stem cell characteristics within the bulk of tumours are implicated in the pathogenesis of heterogeneous malignant tumours. The cells that drive tumour growth have been denoted cancer-initiating cells or cancer stem cells (hereafter CSCs). CSCs have been isolated initially from leukaemias and subsequently from several solid tumours including brain, breast, prostate, colon and lung cancer. This study aimed at isolating and characterising the population of tumour-initiating cells in non-small-cell lung cancer (NSCLC). **Methods:** Specimens of NSCLC obtained from 89 patients undergoing tumour resection at the Cancer National Institute of Naples were analysed. Three methods to isolate the tumour-initiating cells were used: (1) flow cytometry analysis for identification of positive cells for surface markers such as CD24, CD29, CD31, CD34, CD44, CD133 and CD326; (2) Hoechst 33342 dye exclusion test for the identification of a side-population characteristic for the presence of stem cells; (3) non-adherent culture condition able to form spheres with stem cell-like characteristics. Definition of the tumourigenic potential of the cells through soft agar assay and injection into NOD/SCID mice were used to functionally define (*in vitro* and *in vivo*) putative CSCs isolated from NSCLC samples. **Results:** Upon flow cytometry analysis of NSCLC samples, CD133-positive cells were found in 72% of 89 fresh specimens analysed and, on average, represented 6% of the total cells. Moreover, the number of CD133-positive cells increased markedly when the cells, isolated from NSCLC specimens, were grown as spheres in non-adherent culture conditions. Cells from NSCLC, grown as spheres, when assayed in soft agar, give rise to a 3.8-fold larger number of colonies in culture and are more tumourigenic in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice compared with the corresponding adherent cells. **Conclusions:** We have isolated and characterised a population of CD133-positive cells from NSCLC that is able to give rise to spheres and can act as tumour-initiating cells.
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Keywords: Cancer stem cells; CD133; Spheres; Non-small-cell lung cancer (NSCLC)

1. Introduction

Cancer stem cells (CSCs) have been shown to be the source of many solid tumour types including lung carcinoma [1—3]. Lung cancer is the leading cause of cancer deaths in the world, with 1.3 million deaths worldwide annually. Lung cancer can be subdivided into two main subgroups that show major differences in histopathologic and genetic character-istics: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), with the latter comprised of three major histological sub-types: adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large-cell lung cancer (LCLC). As a whole, patients affected by lung cancers have a poor prognosis. The overall 5-year survival rate for lung cancer is <14% [4], for SCLC alone it is even worse, <5% [5]. Such poor prognosis depends on a late diagnosis and a high rate of recurrence, which result in patients presenting an advanced stage of cancer at the diagnosis, for which chemotherapy and radiotherapy have limited efficacy. Therefore, understanding the mechanism underlying lung carcinogenesis is essential for better management of lung cancer patients.

Emerging evidence suggests that specific sub-populations of cancer cells with stem cell characteristics within the bulk of tumours are implicated in the pathogenesis of hetero-geneous malignant tumours [6—9]. The cells that drive tumour

growth have been denoted cancer-initiating cells or cancer stem cells. These have been isolated, initially, from leukaemias and subsequently from several solid tumours including breast, brain, prostate, colon and lung cancer [1—3,10,11]. Similar to their normal counterpart, a key characteristic of CSCs is their capacity for self-renewal and to regenerate the tumour when serially grafted into recipient animals. Moreover, given the inherent resistance of CSCs to drugs, failure of current cancer therapies may be ascribed to the inefficacy of drugs on potentially quiescent CSCs that remain vital and retain their full capacity to reproduce the tumour [12—14]. Treatment strategies for the elimination of cancer, therefore, need to consider the presence of CSCs, though the development of new CSC-targeted strategies is currently hindered by the lack of reliable markers for their identification and the poor understanding of their behaviour and fate determinants. However, CSCs have been prospectively isolated from solid tumours using a variety of stem cell markers, including CD34 [15], CD133 [2] and CD24 often coupled with migration molecules such as CD44 [1], CD29 [10] and CD31 (PECAM) [1—3,10,11] In lung cancer, CSCs have been identified as a subset of cells that extrude Hoechst 33342 dye (side population) [16], such as CD133-positive cells [3] or aldehyde dehydrogenase (DH) positive cells [17]. The aim of the present work was to analyse human non-small-cell lung cancer for expression of CD133 by cytofluorimetric analysis and to isolate and characterise tumour-initiating cells from 89 surgically removed primary NSCLC. Moreover, CD24, CD29 and CD44 were also tested to analyse the migration and metastatic potential of isolated cells. In addition, by culture condition similar to the one used for human neural stem cells [2], we were able to form spheres with stem cell-like characteristics from a commercial NSCLC-stabilised cell lines CALU1, and, from LC12, LC31 and LC52, NSCLC-stabilised cell lines obtained from fresh tumours. Besides, the Hoechst 33342 dye exclusion test was also used to characterise stem cells population as described in several reports [14,16]. Moreover, to verify our hypothesis, NSCLC-stabilised cell lines capable of forming spheres and enriched in CD133-positive cells were also tested for their ability to grow in soft agar, in addition tumourigenesis in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice was compared between spheres and the corresponding adherent cells.

2. Materials and methods

2.1. Cell culture

Specimens of primary NSCLC were obtained from 89 consenting patients undergoing tumour resection and had enrolled in the study from April 2004 to April 2006 at the Cancer National Institute of Naples. Diagnosis was based on clinical and histological parameters (49 adenocarcinomas, 32 epidermoid—squamous carcinomas and eight other histotypes). The patients ranged in age from 29 to 82 years, and 66% were male and 44% female. None of them was subjected to any chemotherapy before surgery. Tumour specimens were minced with scissors and subsequently digested by incubation for 3 h at 37 °C in Iscove's Modified Dulbecco's Medium (IMDM) containing type I collagenase, 1 mg ml^{-1} (Sigma Chemical

Co., St. Louis, MO, USA). After washing in medium plus 10% foetal bovine serum (FBS; Lonza Group Ltd., Basel, Switzerland), the cell suspension was filtered through 40-μm nylon meshes. The cells were plated in different mediums to favour the adhesion and growth of epithelial tumour cells in order to avoid the contamination of the stroma cells: (1) IMDM culture medium plus 10% FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (all purchased from Invitrogen, San Giuliano Milanese, Milan, Italy); (2) bronchiolar epithelial basal medium (BEBM) supplemented with BEGM (pre-packaged SingleQuots containing retinoic acid, bovine pituitary extract, insulin, hydrocortisone, transferrin, triiodotyronine, epinephrine, human epidermal growth factor, gentamicin and amphotericin B (all from Lonza Group Ltd., Basel, Switzerland)) plus 10% FBS, and they were cultured in a humidified incubator at 37 °C under 5% CO_2 atmosphere. All the tumours capable of growing after 9—15 culture passages were reported as lung-stabilised cancer cell lines such as LC12, LC31 and LC52.

The lung carcinoma cell lines, CALU1 and A549, were purchased from the ATCC cell bank.

2.2. Flow cytometric analysis

Adherent growing cells were detached using 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), counted and washed in 0.1% BSA in PBS. At least 200 000 cells were incubated with 1 μg ml^{-1} of fluorescent-labelled monoclonal antibodies or respective isotype controls at 4 °C for 30 min in the dark. After washing, the labelled cells were analysed by flow cytometry using a FACS Vantage cell sorter (Becton & Dickinson, Mountain View, CA, USA). The same procedure was also performed on spheres. The antibodies used were: mouse anti-human CD133/2 PE (575/26 dichroic filter) conjugated, mouse anti-human CD34 FITC (530/30 dichroic filter) and PE conjugated, mouse anti-human CD326 FITC (EpCAM) and PE conjugated, mouse anti-human cytokeratin (CK3-3E4) FITC conjugated and mouse anti-human Ki67 FITC conjugated, all provided by Miltenyi Biotec S.r.l. Calderara di Reno, Bologna, Italy. Mouse anti-human CD24 PE conjugated, mouse anti-human CD29 PE-Cy^{TM}5 (630/22 dichroic filter) conjugated, mouse anti-human CD31 FITC conjugated and mouse anti-human CD44 FITC conjugated, all provided by BD Pharmingen, Buccinasco, Milan, Italy.

For Ki67 intracellular staining, cells were processed using the Caltag Fix & Perm Kit (Invitrogen, Milan, Italy) following the manufacturer's guidelines and labelled with 1 μg ml^{-1} of mouse anti-human Ki67. All data were analysed using CellQuest software.

2.3. Side-population analysis (SPA)

Hoechst 33342 dye exclusion test is one of the methods to isolate stem cells since they are able to exclude drugs, dye and all other substances by over-expression of ABC transporters [16,18]. All cell lines tested as CALU1, A549, LC12, LC31 and LC52 were suspended at 2.0×10^6 cells ml^{-1} in IMDM culture medium pre-warmed at 37 °C and divided into two portions. A portion was treated with 50 μM verapamil, an inhibitor of ABC transporters and the other was left untreated. Both portions were incubated in IMDM

culture medium with 5 μg ml^{-1} Hoechst 33342 (Sigma, Milan, Italy) for 90 min at 37 °C with intermittent stirring. After incubation the cells were washed in PBS and kept on ice for 5 min until analysis by FACS Vantage (Becton Dickinson, Milan, Italy). The Hoechst 33342 dye was excited at 350-nm ultraviolet and the resultant fluorescence was measured at two different wavelengths using a 424/44 BP and 675 LP filters for detection of Hoechst blue and red, respectively. The results were analysed comparing data from verapamil-treated and untreated cells using CellQuest software.

2.4. Non-adherent culture conditions for spheres production

All cell lines tested as CALU1, A549, LC12, LC31 and LC52 were plated at a density of 60 000 cells per well in six-well ultra-low attachment plates (Corning Inc., Corning, NY, USA) in BEBM cell medium, supplemented with BEGM (pre-packaged SingleQuots containing retinoic acid, bovine pituitary extract, insulin, hydrocortisone, transferrin, triio-dotyronine, epinephrine, human epidermal growth factor, gentamicin and amphotericin B (all from Lonza Group Ltd., Basel, Switzerland) plus human EGF (10 ng ml^{-1}; Sigma, Milan, Italy) and human bFGF (10 ng ml^{-1}; Sigma, Milan, Italy). Cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2. Fresh aliquots of EGF and bFGF were added twice a week. After 48—72 h of culture spheres, referred to as pneumospheres, were visible at inverted phase-contrast microscopy. Interestingly, A549 cells were not able to give spheres, though Hoechst 33342 dye exclusion test evidenced a side population and cytometry evidenced CD133-positive cells.

2.5. Immunohistochemistry

Immunohistochemistry for CD133 on CALU1, A549, LC12, LC31 and LC52 cells was performed by plating cells at a density of 100 000 cells per well in 24-well plates, fixed with 3.5% paraformaldehyde for 10 min at 4 °C and washed in PBS. Primary antibody used was mouse anti-human CD133/1 Pure (Miltenyi Biotec S.r.l. Calderara di Reno, Bologna, Italy). The DAKO Cytomation En Vision + System-HRP kit (AEC) was used according to the manufacturer's instructions. This procedure was performed on both adherent cells and pneumospheres.

2.6. Soft agar assay

To evaluate the tumourigenicity of floating spheres versus adherent cells, both types of cells were plated in soft agar at a density of 500, 1000 and 5000 cells per well in 24-well plates, in triplicate. The test was performed using 0.8% and 0.3% agar in IMDM as the base and top layers, respectively.

Spheres and adherent cells, CALU1, LC12, LC31 and LC52, were plated and incubated for 21 days at 37 °C in a humidified atmosphere of 5% CO_2 in air and 50 μl of IMDM culture medium were added twice a week. At the end of the incubation period, colonies were stained with NBT (nitro-bluetetraziolium) at a concentration of 50 mg/100 ml in PBS and counted using an inverted microscope (Nikon TS 100, Milan, Italy).

2.7. Subcutaneous xenografts into NOD/SCID mice

For mice xenografts, cells were mechanically dissociated to obtain single-cell suspensions, diluted in growth factor-containing medium, mixed with matrigel and injected subcutaneously. Six-week-old female NOD/SCID mice were used. Serial dilutions of cells of CALU1 and LC31 (1 and 5×10^4; 1 and 5×10^5; 1 and 5×10^6 cells) were injected to evaluate the tumourigenic activity of NSCLC sphere-derived cells. Mice were monitored every 3 days for the appearance of subcutaneous tumours. When tumour diameters reached 10 mm in size, mice were sacrificed and the tumour tissue collected, fixed in buffered formalin and subsequently analysed by immunohistochemistry. Haematoxylin and eosin staining followed by immunohistochemical analysis were performed to analyse tumour histology. The injection experiments were in triplicate.

2.8. Correlation between CD133 and clinical parameters

To correlate the presence of CD133-positive cells with the outcome of diseases, the following clinical parameters were analysed: age, sex, histotype and clinical stage. Moreover, since all the 89 patients enrolled in the study had a follow-up of at least 2 years, outcome of the disease was reported as patients with no evidence of the disease (NED) after 2 years of follow-up and patients with progression of the disease (PD) when metastatic recurrence was observed.

2.9. Statistical methods

Correlation between presence of CD133-positive cells and clinico-pathological parameters has been analysed by Fisher's exact test. Levels of significance were set at $p < 0.05$.

3. Results

3.1. Flow cytometric analysis

Fresh surgical biopsies of NSCLC obtained from 89 patients undergoing tumour resection were mechanically/enzymatically disaggregated as reported in Section 2 and the resulting single-cell suspensions were analysed by flow cytometry to identify the phenotypic characteristics of different cell populations. In order to investigate the possible presence of a stem-/initiating-cell population, we analysed the expression of CD24, CD29, CD31, CD34, CD44, CD133 and CD326 antigens on single-cell suspensions. An example of experiment is reported in Fig. 1, where the results showed that cells positive for CD133 represented 16.2% of the total cells population and only 6.5% of the cells expressed CD34 antigen. Moreover, CD24-, CD29- and CD44-positive cells were 9.7%, 8.9% and 23.7%, respectively. The fraction of endothelial cells, CD31 positive, was always less than 2% of the total population. Besides, we have found a first sub-population CD34 and CD326 double positive, representing 11.7% of the total cells analysed and a second cell-subset positive for both CD133 and CD326, representing 8.3% of the total cells analysed. We have focussed on this last population since probably it represents NSCLC cancer-initiating cells. In addition, flow cytometric
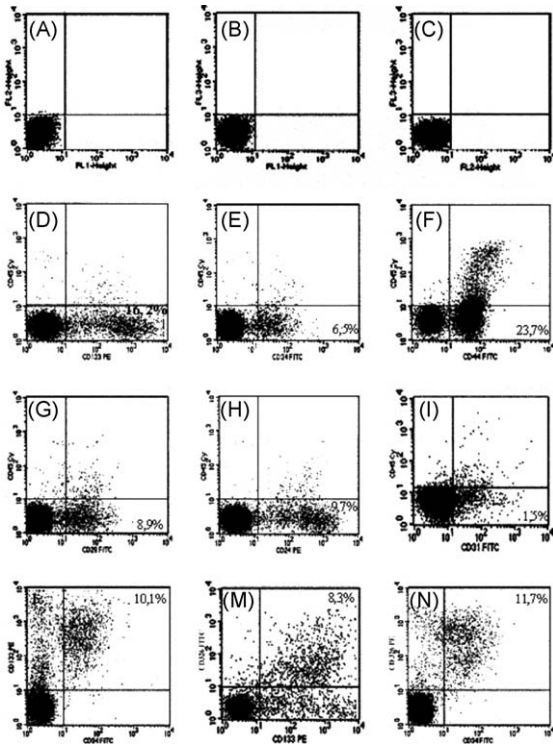
Fig. 1. Flow cytometric analysis of a representative example of different stem markers expressed on cells present in fresh biopsies. Panels A, B, C: isotype control; Panel D: CD133+ cells represented 16.2%; Panel E: CD34+ cells represented 6.5%; Panel F: CD44+ cells represented 23.7%; Panel G: CD29+ cells represented 8.9%; Panel H: CD24+ cells represented 9.7%; Panel I: CD31+ cells represented 1.5%; Panel L: CD34+CD133+ cells represented 10.1%; Panel M: CD133+CD326+ cells represented 8.3%; Panel N: CD34+CD326+ cells represented 11.7%.

analysis reported in Table 1 showed that CD133 was expressed on 72%, CD34 on 82.0%, CD44 on 94.3%, CD29 on 88.7% and CD24 on 84.2% of the total 89 fresh specimens analysed.

Presence of CD133-positive cells was also tested in adherent NSCLC-stabilised cell lines CALU1, A549, LC12, LC31 and LC52 and the expression levels ranged from 1% to 10% (Fig. 2).

3.2. Cell culture from NSCLC specimens

We had obtained three stabilised cell lines by 89 tissue specimens after enzymatic digestion. The three stabilised cell lines were called LC12, LC31 and LC52. These were grown in IMDM plus 10% FBS and derived from patients affected by adenocarcinoma, epithelioid—squamous carcinoma and neuroendocrine carcinoma, respectively. All three cell lines obtained were positive for cytokeratins 8, 18, 19 by flow cytometry. We found that the expression levels of CD133 were 9.5%, 5.05% and 1.35% for LC12, LC31 and LC52, respectively (Fig. 2).

3.3. Spheres analysis and proliferation assay

Since sphere assay has proven to be an excellent technique to isolate stem cells and progenitor cells, we used non-adherent culture condition to form spheres with stem cell-like characteristics starting from NSCLC-stabilised cell lines as CALU1, A549, LC12, LC31 and LC52. Floating spheres were already observed after 24—48 h of culture in FBS-free medium supplemented with EGF and bFGF (Fig. 3, Panels B and C). These spheres can be dissociated and expanded for at least 10 generations and they can be frozen in liquid nitrogen as well. Using the usual cell culture conditions, the floating spheres can become adherent and assume an epithelial shape. As reported in Section 2, A549 cells were not able to produce spheres, though Hoechst 33342 dye exclusion test evidenced a side population and cytometry evidenced CD133-positive cells.

Spheres were analysed for expression of CD133 antigen and they were found to be enriched with CD133 compared to the same cells growing in adherent condition. As reported for CALU1 cells, in Fig. 4, Panels A and D, the expression of CD133 is four times higher in floating spheres (48.02%) with respect to the same cells growing in adherent conditions (10.07%). Panel E reports the histogram of the experiment described above.

Since Ki67 is a nuclear protein that is expressed from the cells in late G1 and in G2M phases of the cell cycle, the expression of this antigen was used to determine whether

Table 1
Distribution of stemness and differentiation markers in NSCLC 89 total fresh specimens and markers background.

| Markers	Mean percentage of positive cells % (range)	Percentage of positive fresh specimens N (%)	Markers background
CD133	6% (0.23—16.20)	64 (71.9%)	CD133 antigen is a transmembrane molecule expressed on hematopoietic stem cells, on circulating endothelial progenitor cells, neural stem cells, renal and prostate stem cells [7].
CD34	4% (0.21—10.68)	73 (82.0%)	CD34 is an adhesion molecule expressed on human hematopoietic stem and progenitor cells, endothelial progenitor cells and vascular endothelial cells [10].
CD44	9% (0.25—24.61)	84 (94.3%)	CD44 is a cell adhesion receptor, and its ligands are hyaluronate and the cytokine osteopontin. CD44 is expressed in lymphoid, mieloid and erythroid cells, mesenchymal stem cells and may be useful predictor of lymph node metastasis [6].
CD29	12% (0.21—29.58)	79 (88.7%)	CD29 is a beta1 integrin involved in cell adhesion embryogenesis, tissue repair, immune response and metatastatic diffusion of tumor cells reacting with thrombocytes, monocytes and a T and B lymphocytes and is also expressed on mesenchymal stem cells [9].
CD24	10% (0.27—15.78)	75 (84.2%)	CD24 antigen is a glycosylphosphatidylinositol-linked membrane sialoglycoprotein. CD24 is present on B cells, from the stage pre-B to the mature B cell stage, but not on plasma cells. It is expressed on mature granulocytes and on a variety of epithelial cell types [6]
CD133CD326	5% (0.28—9.51)	62 (69.6%)	CD326 is an adhesion molecule called EpCAM expressed on the basolateral surface of carcinoma and
CD34CD326	3% (0.27—11.17)	63 (70.7)%	epithelial cells.
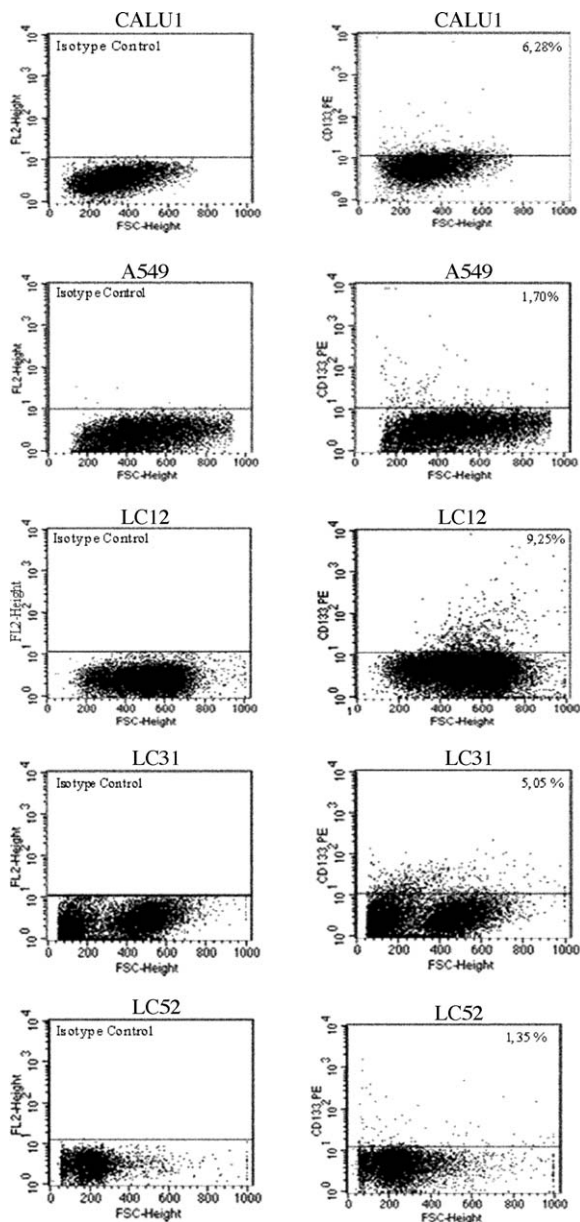
Fig. 2. Presence of CD133-positive cells was tested in adherent NSCLC-stabilised cell lines CALU1, A549, LC12, LC31, LC52. Expression levels ranged from 1% to 10%.
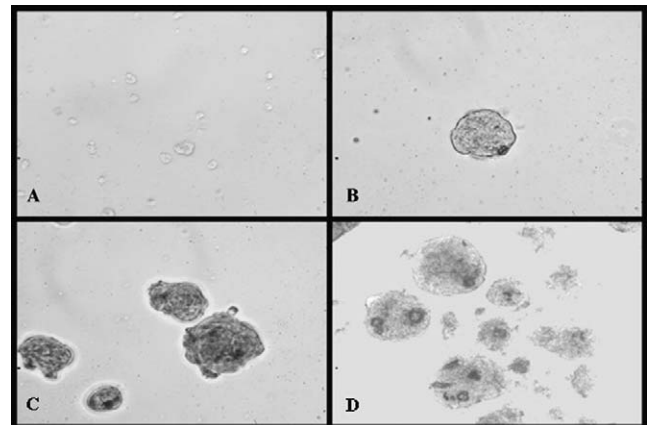

Fig. 3. CALU1 cells growing in non-adherent conditions. Panels A, B, C, D report T0, 24 h, 48 h and 72 h, respectively.

anchorage-independent growth properties of NSCLC cells growth as spheres or in adherent conditions, we performed a soft agar assay as described in Section 2 using cells from dissociated spheres obtained from cell lines CALU1, LC12, LC31 and LC52. We found that after 21 days of incubation,
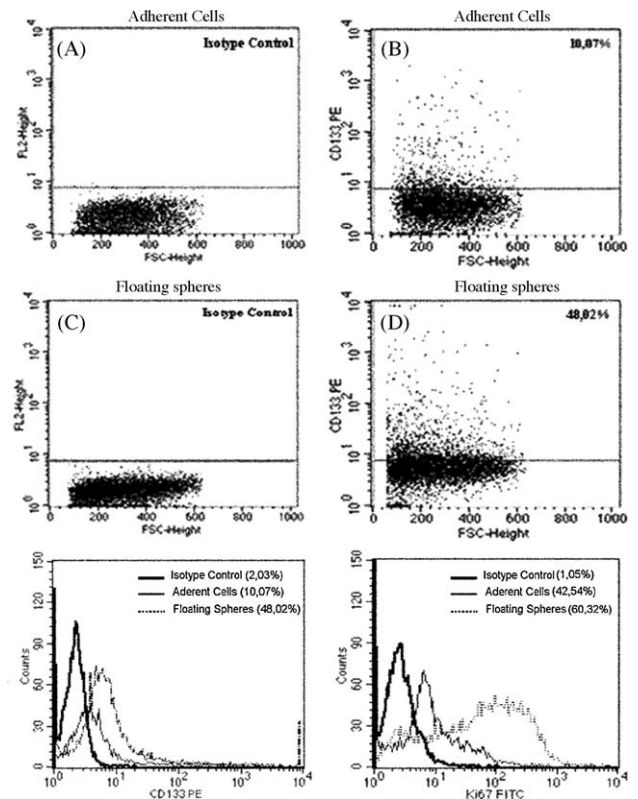

Fig. 4. Cytometric CD133 expression in CALU1 both in adherent cells (A, B) and in floating spheres (C, D). The spheres were enriched of CD133 antigen compared with adherent counterpart. The quantity of CD133 had a 4-fold higher expression in floating spheres (48.02%) in respect to same adherent cells (10.07%). Panel E: histogram plot showing different expression of CD133 antigen in spheres and adherent cells. Panel F: histogram plot showing different proliferation rate in spheres and adherent cells. The floating spheres expressed Ki67 in mean percentage of 60.32% in respect to 42.54% of adherent cells.

there were differences in the proliferation of floating spheres compared to the adherent cells. Results, referred to as CALU1 cells and reported in the histogram of Fig. 4, Panel F, revealed that the floating spheres expressed Ki67 in a mean percentage of 60.32% compared to 42.54% of adherent cells. Our data indicate that the floating spheres were in active proliferation compared to the adherent cells.

3.4. Sphere-derived cells showed increased anchorage-independent growth in soft agar

One of the methods of analysing the transformed phenotype of the cells is the soft agar assay that measures anchorage-independent growth, which is an indicator for assessing cell transformation. In order to assess the
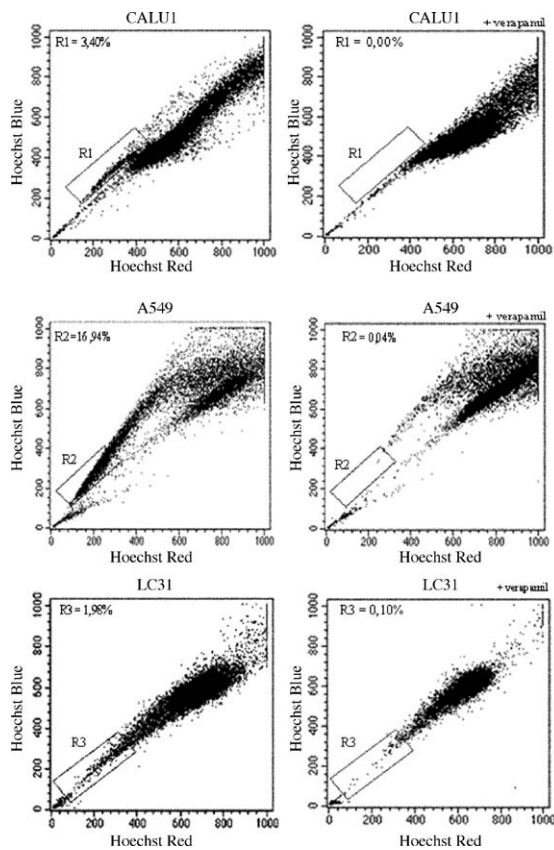
Fig. 5. Side population in CALU1, A549 and LC31. Side population cells comprise 3—5% of total population in CALU1, 10—15% in A549 and 1—3% in LC31.
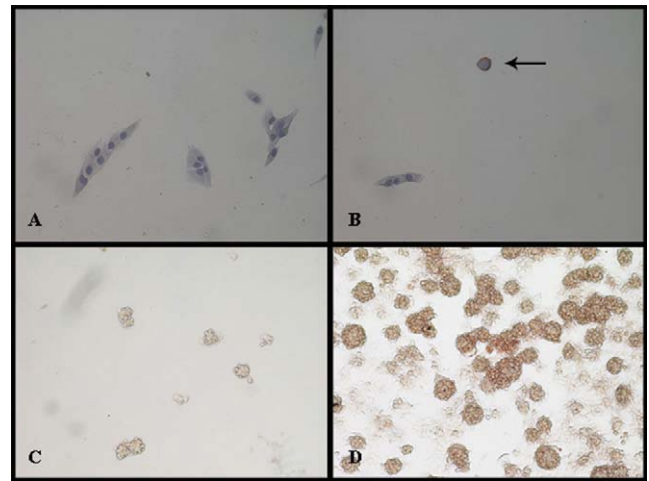

Fig. 6. Cd133 expression in CALU1 both in spheres and adherent cells. Panel A: isotype control; Panel B: CD133-positive cell in adherent culture condition (arrow); Panel C: isotype control; Panel D: CD133 expression in floating spheres that showed a diffuse staining for CD133, confirming that spheres are enriched by CD133⁺ cells.

spheres gave rise to an increased number of colonies (on average 3.8-fold larger) than those observed in adherent cells: i.e. 3.5 ± 1.2-fold larger in CALU1 ($p < 0.005$); 5.2 ± 1.5-fold in LC12 ($p < 0.001$), 3.8 ± 1.1-fold in LC31 ($p < 0.005$) and 2.8 ± 1.3-fold in LC52 ($p < 0.005$). More-over, colonies originating from dissociated spheres were larger than those originating from single adherent cells.

3.5. Side-population analysis

Hoechst 33342 dye exclusion test for the identification of a side-population characteristic for the presence of stem cells was used to analyse NSCLC-stabilised cell lines CALU1, A549 and LC31. Data were reported comparing verapamil-treated and untreated cells. Results reported in Fig. 5 showed that SP cells in CALU1, A549 and LC31 were 3.40%, 16.94% and 1.98% in untreated cells in comparison to 0.0%, 0.04% and 0.10% in verapamil-treated cells, respectively.

3.6. Immunohistochemistry

An immunohistochemical staining was performed to investigate the localisation and different distribution of CD133 antigen both on adherent cells and floating spheres. Floating spheres showed a diffuse staining for CD133 (Fig. 6, Panels C and D), confirming that spheres are enriched by CD133⁺ cells whereas a faint positivity of the CD133 antigen was found on the cell membrane of adherent cells (Fig. 6, Panels A and B).

3.7. Sphere-derived cells showed increased tumourigenicity in NOD/SCID mice

We evaluated the tumourigenic potential of NSCLC cells in NOD/SCID mice through subcutaneous injection of lung sphere cells mixed with growth factor-reduced matrigel. The injection of as low as 10^4 sphere-derived cells of CALU1 and LC31 consistently resulted in the growth of tumour xenografts with morphological features closely resembling those of the original tumour, as shown by haematoxylin and eosin staining. Large lesions formed within 50 days. Complete similarity between patient tumour and mouse xenograft was found, demonstrating that tumour spheres could effectively reproduce the human disease in mouse.

3.8. Correlation between CD133 and clinical parameters

It has been hypothesised that the CSCs content in tumour may correlate with the more aggressive clinical—pathological features of the disease, and clinical parameters and outcome of the disease was determined as described in Section 2. Data are presented in Table 2 and apparently no statistically significant correlation was found with any of the parameters considered. However, it has to be noted that, using cytometry, CD133 antigen has been found to be strongly expressed in poorly differentiated tumours, and it seems to be more frequent in patients with no evidence of disease (76%) compared to patients with disease progression (55%) with $p < 0.07$. A longer follow-up is needed before drawing definitive conclusions.

4. Discussion

The hypothesis that the tumours originate from a small-cell subset with stem cell characteristics has been demon-strated by different studies in breast, brain, prostate and other solid tumours [1,2,10]. Stem cells are functionally defined as self-renewing, quiescent and multi-potent cells

Table 2
Clinico-pathological characteristics and expression of CD133 in primary NSCLC.

Variable	No. of patients	No. of patients with CD133-positive cell samples	p value
Total	89	64 (72%)	
Tumour extension			
pT1-pT2	71	51 (72%)	
pT3-pT4	18	13 (72%)	0.61
Lymph node involvement			
pN0	75	55 (73%)	
pN1-pN2	14	9 (64%)	0.34
Tumour histology			
Adenocarcinoma	49	35 (71%)	
Epidermoid—squamous carcinoma	32	23 (71%)	0.58
Other histotypes	8	6 (75%)	
Follow-up			
NED	71	54 (76%)	
PD	18	10 (55%)	0.07
Age			
<60 years	33	22 (67%)	
>60 years	56	42 (75%)	0.27
Sex			
Female	30	25 (83%)	
Male	59	39 (66%)	0.06

that exhibit multi-lineage differentiation. In haematological malignancies such as leukaemia, multiple myeloma and in solid tumours, rare cells were isolated with a remarkable potential for self-renewal and these cells alone were found to drive the formation of the original tumours in NOD/SCID mice [1,2].

In this study, we investigated whether NSCLC could contain tumour cell subsets that could be defined as CSCs or cancer-initiating cells. For this purpose, we have analysed the presence of CD133 antigen both in fresh human NSCLC specimens and in stabilised cell lines. In agreement with the general concept that CSCs are only a minimal part of the total tumour cell population, we found that the CD133 was expressed in a very small percentage of the total cell population of NSCLC. CD133 was found expressed in 71.9% of the total fresh specimens analysed with a mean percentage of 6%, supporting the hypothesis of the existence of CSCs in NSCLC. In addition, it is interesting to note that CD34 antigen was found to be expressed in the same specimens as well. Our data showed that the vast majority of specimens analysed had CD133+ or CD34+ cells, both positive for CD326, confirming their epithelial origin. As a consequence, we hypothesised that two cell sub-populations existed in fresh biopsies: one CD133+CD326+ and the other CD34+CD326+, which can be both considered as distinctive of stem—progenitor cells. In this context, we can hypothesise that the CD133 and CD34 expression concerned not only stem cells but also committed progenitors downstream in the tumour cell hierarchy. Interestingly, in stabilised cell lines of NSCLC, such as CALU1, A549, LC12, LC31 and LC52, we found onlyCD133+CD326+, thus indicating that probably this population was the real stem cell population. We have demonstrated, indeed, that when the cells were growing in an adherent condition, the CD133 expression was limited to 1—

10%, whereas the same cells, growing in serum-free medium with bFGF and EGF and forming spheres enriched with CD133 antigen, reached 40%. These spheres can be dissociated and expanded for at least 10 generations. Besides, we found that they exhibited more extensive proliferative potential compared to adherent cells evidenced by expression of Ki67. In addition, the floating spheres have been grown in soft agar and formed colonies with an efficiency 3.8% greater than that adherent cells. Furthermore, the floating spheres were highly tumourigenic due to their capacity to form an original human tumour when injected in NOD/SCID mice with respect to corresponding adherent cell lines. Moreover, side-population test was also performed using Hoechst 33342 dye exclusion test and cell lines were all positive, although side population represents only 1—15% of the total cells analysed. All these data demonstrated that fresh tumours and stabilised cell lines contained stem cell-like population, which were highly tumourigenic.

In conclusion, our study showed new important insights into NSCLC. In particular, we have identified CD133 antigen as a potential lung cancer stem/initiating-cell marker. In fact, we have demonstrated the presence of CD133+ cells both in fresh biopsies of NSCLC and in stabilised cell lines. These cells, also growing as spheres, are highly tumourigenic, proliferative and enriched in CD133-positive cells. As reported for other solid tumours [1—3,10,11,15], these cells can represent the cancer-initiating cells capable of giving rise to primary tumour growth, invasion and spread as distant metastases. As a consequence, presence of CD133 antigen could be an important key to study the biology and behaviour of lung cancers and can represent an important and useful tool to facilitate the development of new diagnostic and prognostic procedures. For these reasons, in this study, clinical parameters and presence of CD133 were also reported, although no statistically significance correlation was found. This discrepancy can be probably explained by the short time of follow-up. As a consequence, a longer period of study is needed to determine if targeting of this cell population would be critical for therapeutic success.

Acknowledgements

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