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ORIGINAL ARTICLE

CD133-Positive Cells from Non-Small Cell Lung Cancer Show Distinct Sensitivity to Cisplatin and Afatinib

Angela Alama · Rosaria Gangemi · Silvano Ferrini · Gaia Barisione · Anna Maria Orengo · Mauro Truini · Maria Giovanna Dal Bello · Francesco Grossi

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Abstract The standard of care for advanced non-small cell lung cancer (NSCLC) consists in cisplatin-combination chemotherapy. In patients bearing tumors with activating mutations of the epidermal growth factor receptor (EGFR), the inhibition of the EGFR intracellular tyrosine kinase can induce up to 80 % response rates. However, both therapeutic strategies will eventually lead to recurrent disease due to the development of drug resistance. The identification of rare cancer stem-like cells able to repopulate the tumor, after failure to standard treatment modalities, has led to characterize these cells as potential therapeutic targets. This article will address the role of the CD133/EpCAM stem cell-related markers and explore cell sensitivity to cisplatin and to the EGFR-tyrosine kinase inhibitor, afatinib. Three human NSCLC cell lines, one wild-type (A549) and two harboring *EGFR* mutations (H1650 and H1975), as well as 20 NSCLC primary cultures, were grown in non-differentiating culture conditions for stem cell enrichment. Flow-cytometry analyses of CD133 and EpCAM and cell sensitivity to cisplatin and afatinib were performed. Moreover, the expression of activated EGFR was assessed by Western blot. The cell lines and primary cultures grown in non-differentiating culture conditions were enriched with CD133/EpCAM-positive cells and were significantly more resistant to cisplatin and more sensitive to afatinib as compared to the differentiated counterpart. In addition, increased EGFR-phosphorylation in non-differentiated cultures was observed. The present findings suggest that afatinib might be beneficial for patients bearing tumors with constitutively activated EGFR, to target chemo-resistant CD133/EpCAM-positive cancer stem cells.

Keywords NSCLC · CD133 · EpCAM · Cisplatin · Afatinib · Drug-resistance

A. Alama and R. Gangemi equally contributed to this work.

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A. Alama (✉) · M. G. D. Bello · F. Grossi
Lung Cancer Unit, IRCCS A.O.U. San Martino-IST, National Institute for Cancer Research, Largo Rosanna Benzi, 10, 16132 Genoa, Italy
e-mail: angela.alama@hsanmartino.it

R. Gangemi · S. Ferrini · G. Barisione · A. M. Orengo
Laboratory of Bio-therapy, IRCCS A.O.U. San Martino-IST, National Institute for Cancer Research, Largo Rosanna Benzi, 10, 16132 Genoa, Italy

M. Truini
Department of Pathology, IRCCS A.O.U. San Martino-IST, National Institute for Cancer Research, Largo Rosanna Benzi, 10, 16132 Genoa, Italy

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death in both men and women in the US and accounts for approximately 85 % of all cases of lung cancer (Ferlay et al. 2010). The treatment of NSCLC is determined by stage, being surgery the treatment of choice for early and localized disease. Multimodal cisplatin-based chemotherapy has become the standard of care for patients with advanced and metastatic neoplasms (Cufer et al. 2013).

Recent advances in lung cancer biology revealed a number of molecular markers, which can be potentially targeted by novel agents. Among all, activating epidermal

growth factor receptor (*EGFR*) gene mutations emerged as the most relevant predictors of response to treatment with EGFR-tyrosine kinase inhibitors (EGFR-TKIs). Indeed, different studies indicated that EGFR-TKIs, such as gefitinib and erlotinib, induce response rates up to 80 % in patients carrying *EGFR* mutations in their tumors (Ono and Kuwano 2006; Pao et al. 2004). Many types of mutations have been described, but only five seemed to be significantly involved in EGFR-TKI sensitivity: exon 19 deletion (E746A750), exon 21 L858R substitution, exon 18 (G719A/C) and exon 21 (L861Q) substitutions together with a secondary exon 21 mutation (L858R/exon20 T790M), mainly involved in gefitinib and erlotinib resistance. These mutations are more commonly associated with patients showing specific features: Asians, women, non-smokers and adenocarcinoma histology (Pao et al. 2004).

Although a number of patients with NSCLC will experience striking response to cisplatin-based chemotherapy or EGFR-TKI treatment, others will eventually relapse for the development of drug resistance and, in a minority of cases, both therapeutic strategies will be unsuccessful ab initio (Chang 2011; Chen et al. 2010; Nguyen et al. 2009). Irreversible EGFR-TKIs, such as afatinib (BIBW-2992), have been developed to be effective in inhibiting the growth of the *EGFR*-mutated NSCLC cells resistant to gefitinib or erlotinib in vitro and in vivo (Li et al. 2008).

The frequent failures of current therapies suggest the presence of sub-populations of cancer cells, which escape eradication, and repopulate the tumor leading to disease recurrence and metastasis (Maugeri-Saccà et al. 2011; Tan et al. 2010). These rare tumor cells, unresponsive to standard therapies, were called cancer stem cells (CSCs) for their ability to undergo self-renewal and develop into phenotypically different tumor cell populations that extensively proliferate (Nguyen et al. 2012).

In recent years, the identification of markers preferentially expressed on CSCs, has allowed to isolate and characterize CSCs from tumors to be used as potential therapeutic targets (Alama et al. 2012).

Surface molecules such as CD133 and EpCAM have been widely described as major markers of CSCs, which play an important role in lung cancer progression (Munz et al. 2009; Neuzil et al. 2007; Skirecki et al. 2014). This article will address the role of the CD133/EPCAM-enriched CSC-like cells in drug resistance of NSCLC. To this end, human NSCLC cell lines and primary cultures from fresh NSCLC tissues were grown in non-differentiating or standard culture conditions and were evaluated for CD133 and EpCAM phenotype and sensitivity to cisplatin and afatinib.

Materials and Methods

Cell Lines and Culture Conditions

Three human NSCLC cell lines, one wild-type (WT) and two with activating mutations of *EGFR*, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and used for in vitro studies: A549 (*EGFR-WT, RAS*-mutated), H1650 (*EGFR* exon19 Del E746A750) and H1975 (*EGFR* exon21 L858R; exon20 T790M). Cells were grown in 25 cm^2 flasks, in adherent conditions, in RPMI 1640 medium supplemented with 5 % fetal bovine serum (R/FBS). DMEM-F12 supplemented with B-27 (Gibco® Life Technologies, Europe BV, Italy) plus 20 ng/ml of epidermal growth factor and basic fibroblast growth factor (D/F12) was used for non-differentiating culture conditions. All reagents were from Sigma (St. Louis, MO) and Life Technologies (Europe BV, Italy) unless otherwise specified.

Collection and Processing of Surgically Resected Tumor Samples

Fresh lung cancer tissues from 20 patients with histologically proven stage I–IIIA NSCLC (15 adenocarcinomas and 5 squamous carcinomas), who had undergone surgical resection at the National Institute for Cancer Research (Genova, Italy) were obtained by the pathologist at the time of surgical excision and immediately processed for histological evaluation and cell culture. The patients' median age was 69 years (ranged 55–83), 70 % were male and 30 % female. All the patients' tumors had *EGFR-WT* and none of them underwent chemotherapy before surgery.

Cell suspensions were obtained by mechanical disaggregation of the tumor samples, washed twice in phosphate-buffered saline and plated in 25 cm^2 flasks in R/FBS or D/F12 as described for the NSCLC cell lines. Cultures in R/FBS were excluded whenever a high contamination by fibroblasts was found.

The study was done in compliance with the principle of the Declaration of Helsinki and written informed consent for use of tissue in biological and molecular analyses was acquired from patients at the time of first outpatient visit at IRCCS A.O.U. San Martino-IST, National Institute for Cancer Research in Genova (Italy).

MTT Colorimetric Assay

The colorimetric MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma St. Louis, MO) was used to evaluate the sensitivity of tumor cells to different concentrations of cisplatin (Sigma) or afatinib (BIBW-2992, Selleckchem, USA). Cells were seeded in 96-well plates at

concentrations ranging from 1×10^3 to 6×10^3 cells/well, exposed to the tested compound for 72 h and the viable cell content measured by a standard MTT assay.

All data points represent an average of at least three assays. The mean and standard deviation (SD) from quadruplicate samples were determined for each drug concentration.

Western Blot Analysis

Cell extracts were prepared in RIPA buffer, containing phosphatases and protease inhibitors (Sigma). Protein samples (40 μg) were separated by pre-casted 4–12 % gradient SDS-PAGE and transferred to a nitrocellulose membrane (Life Technologies, Europe BV). Membranes were probed with the appropriate antibodies recognizing EGFR, phosphorylated-EGFR (Tyr1068) or β-Actin. Primary antibodies were detected by using horseradish peroxidase-linked secondary antibodies. All the antibodies were purchased from Life Technologies and Cell Signaling (Cell Signaling Technology, Inc. Danvers, MA). Immunoreactive proteins were visualized by LiteA*blot* TURBO (EuroClone S.p.a.Italy).

Flow-Cytometry

For flow-cytometry analysis, cultured or freshly dissociated cells were stained with the appropriate amount of PE or FITC-conjugated antibodies or PE or FITC-conjugated isotype-specific control antibody. Cells were stained with the following antibodies: anti-CD133 and anti-EpCAM, (BD Bioscience, San Jose, CA). Ten thousand labeled cells were acquired and analyzed using a FACScan flow cytometer running CellQuest software (Becton–Dickinson and Co, Mountain View, CA).

Statistical Analysis

Statistical analysis was performed using an unpaired two-tailed Student's *t* test. Correlation analysis between normally distributed variables was performed using the Pearson test. To test if the values came from a Gaussian distribution, the D'Agostino-Pearson omnibus normality test was used (Prism 5 software). Differences were considered statistically significant at $p < 0.05$.

Results

Phenotypic Analyses of Human NSCLC Cells Maintained in Differentiating or Non-Differentiating Culture Conditions

Three lung cancer cell lines exhibiting *WT* (A549) and mutated *EGFR* (H1650 and H1975) were grown in non-differentiating (i.e. stem cell-like) culture medium (D/F12) or in standard differentiating culture conditions (R/FBS) for 2–6 weeks. Cultures were then assessed for CD133 and EpCAM expression by flow-cytometry analysis.

The A549 and H1975 cells formed floating spheres while the H1650 showed both loosely adherent and sphere-forming populations when cultured in D/F12. As shown in Fig. 1, CD133 and EpCAM expression increased in all cell lines maintained in non-differentiating conditions relative to cells maintained in R/FBS, suggesting that a population expressing stem cell-related markers was enriched in D/F12. In particular, EpCAM appeared more elevated in the *EGFR*-mutated cell lines as compared to the *EGFR-WT* cells, while CD133 expression was higher in the *WT* than in the mutated cells regardless of culture conditions. Two-color immunofluorescence analysis indicated that CD133 and EpCAM-positive cell populations are only partially overlapping (Fig. 1). Real-time RT-PCR showed that CD133 induction in D/F12 culture conditions is related to an increase in *CD133* mRNA expression relative to standard conditions, while the induction of EPCAM may relate to post-transcriptional events as mRNA expression showed no significant changes (Suppl. Fig. 1).

Next, fresh lung cancer tissues from 20 patients undergoing surgical excision for NSCLC were obtained and maintained in non-differentiating culture medium or standard differentiating culture conditions for at least 2 weeks and then assayed for flow-cytometry phenotyping. Analyses have been only feasible in non-adherent sphere cultures due to a limited number of available cancer cells cultured in standard conditions. A summary of flow-cytometry analyses from primary tumor samples is depicted in Fig. 2.

The number of CD133-positive cells was variable among the different samples with a median value of 5.1 % within a range of 0.1–19.3 % while EpCAM expression was extremely variable in all the samples investigated, exhibiting a median value of 54 % (range 1.0–93 %). In general, these observations indicated that culturing primary NSCLC cells in D/F12 resulted in the outgrowth of a sphere-forming subpopulation showing a predominant expression of EpCAM relative to CD133.

The proportions of CD133-positive or EpCAM-positive cultured NSCLC cells showed no significant correlation with patients overall survival (data not shown). However, CD133 expression correlated with cell proliferation in culture (Suppl. Fig. 2).

Subcutaneous injection of representative D/F12 cultures from different patients produced tumor growth in three out of four cases, suggesting that most cells kept under non-differentiating conditions have a tumorigenic potential (Suppl. Fig. 3).

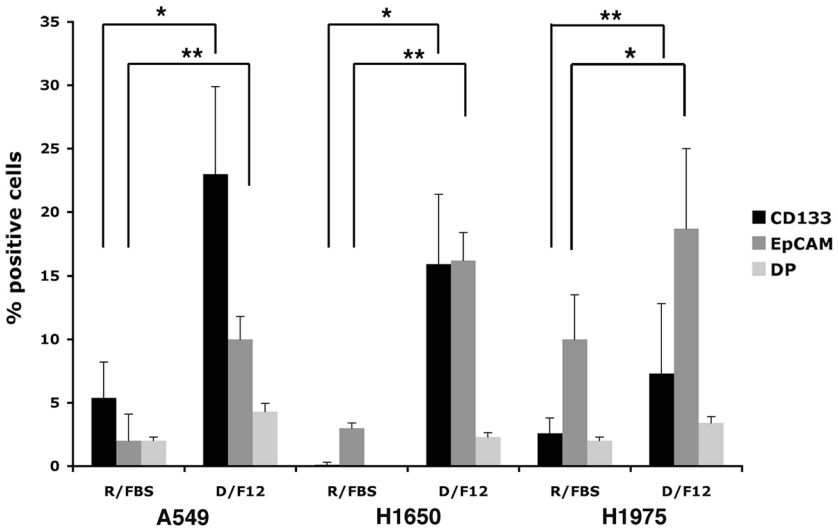

Fig. 1 Expression of EpCAM and CD133 by flow-cytometry in NSCLC cell lines maintained in R/FBS or D/F12 culture conditions. Significant enrichment in CD133/EpCAM and double positive (DP) populations was obtained in the cell lines grown in non-differentiating conditions compared to the differentiated counterparts. Data represent mean values of three independent experiments (*$p < 0.05$; **$p < 0.001$ by Student's t test)

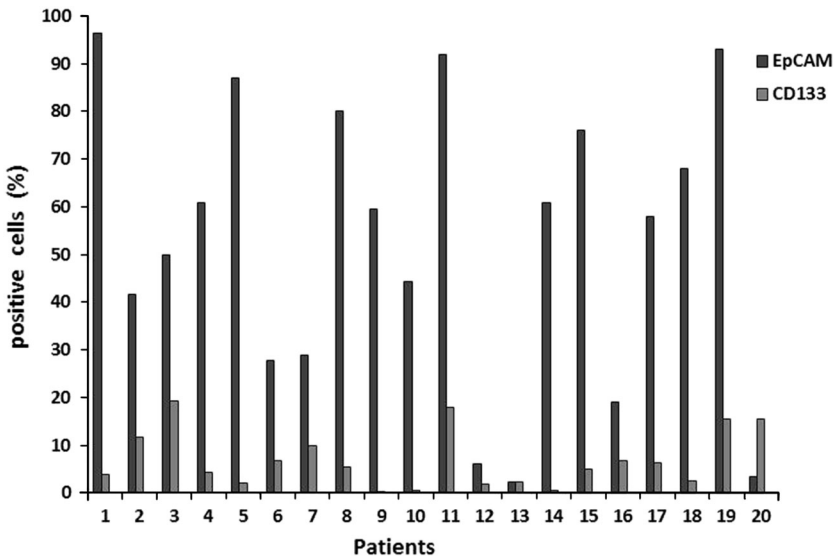

Fig. 2 EpCAM and CD133 phenotyping of 20 samples from NSCLC patients. Cell suspensions from dissociated tumor tissues were grown in D/F12 non-differentiating conditions and then assessed for stem-related markers by flow-cytometry. EpCAM was higher than CD133 expression ($p < 0.001$) by Student's t test

Sensitivity to Cisplatin (CDDP) and Afatinib of NSCLC Cells in Different Culture Conditions

Drug sensitivity of NSCLC cells cultured in D/F12 or R/FBS was tested in the presence of increasing concentrations of CDDP (1.0–20 μM) or afatinib (0.6–10 μM) and anti-proliferative activity was evaluated by MTT. In the first set of experiments, the NSCLC cell lines were evaluated, and the IC_{50} values of CDDP or afatinib were calculated (Table 1). All cell lines grown in D/F12 were significantly more resistant to CCDP than cells maintained in R/FBS. On the other hand, the *EGFR*-mutated H1650 and H1975 cell lines grown in D/F12 were significantly more sensitive to afatinib. Conversely, R/FBS and D/F12-cultured A549 cells showed no difference in their sensitivity to afatinib.

Furthermore, the A549 and H1975 cells, surviving after exposure to CDDP, showed an increased CD133 expression (Fig. 3 and Suppl. Fig. 4), whereas no substantial change was observed in the same cells treated with afatinib. In addition, EpCAM expression showed no significant variations in CCDP- or afatinib-treated cells (Fig. 3). Collectively, these data suggest that the CD133-positive subpopulation is resistant to CDDP treatment and is selected after in vitro treatment with CDDP.

The cytotoxic activity exerted by CDDP was further evaluated in 17 primary cultures maintained in R/FBS or D/F12. A test dose of 20 μM CDDP, determined in a preliminary dose/response assay, was used for all samples. As shown in Fig. 4, most (15 out of 17; 82 %) D/F12-cultured tumor samples showed a lower sensitivity to CDDP than the corresponding R/FBS cultures

Table 1 Drug sensitivity of NSCLC cell lines grown in R/FBS or D/F12

Cell lines	Cisplatin IC_{50} (μM) ± SD	Afatinib IC_{50} (μM) ± SD
A549 R/FBS	7.0 ± 0.03**	3.9 ± 0.06[§]
A549 D/F12	14.2 ± 0.04	3.7 ± 0,07
H1650 R/FBS	11.5 ± 0.03*	4.9 ± 0.04*
H1650 D/F12	18.0 ± 0.08	2.4 ± 0.04
H1975 R/FBS	2.7 ± 0.04**	2.9 ± 0.03**
H1975 D/F12	7.2 ± 0.02	0.6 ± 0.04

R/FBS vs. D/F12: [§] Not significant; * $p < 0.05$; ** $p < 0.005$

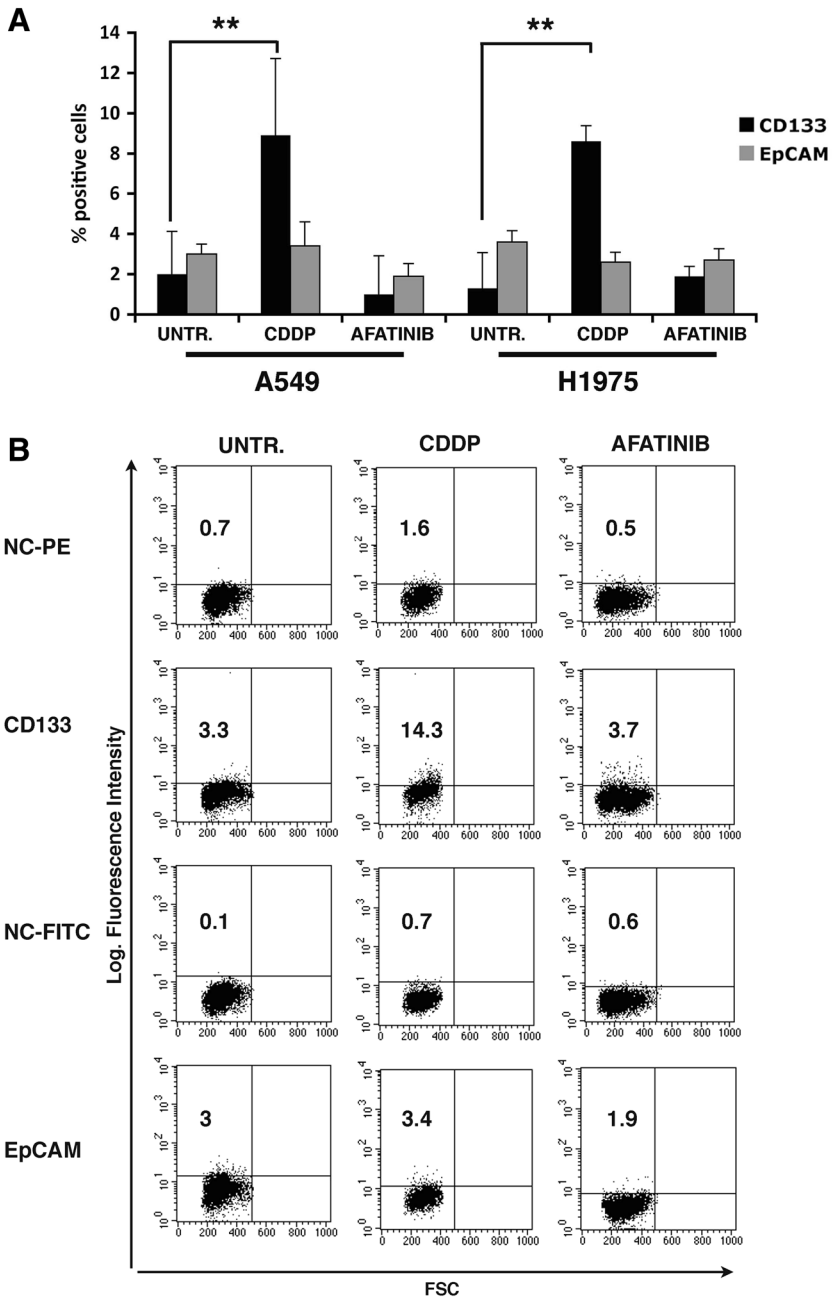

Fig. 3 CD133 phenotype modulation in A549 and H1975 cells cultured in R/FBS and treated with CDDP or afatinib. **a** Histogram showing percentages of cells expressing CD133 or EpCAM by flow-cytometry analysis upon treatment with CDDP or afatinib or drug-free medium (UNTR). The data represent the means of at least three independent experiments $^{**}p < 0.001$. **b** A representative experiment ($n = 3$) of flow-cytometry dot plot analysis of A549 cells is shown: CD133- or EpCAM-positivity is along the *Y*-axis (log. fluorescence intensity). The percentage of positive cells is indicated at the upper right quadrant of each panel. Forward scatter (FSC) is shown along the *X*-axis. NC-PE and NC-FITC indicate controls in the presence of isotype-matched fluorochrome-labeled irrelevant antibodies

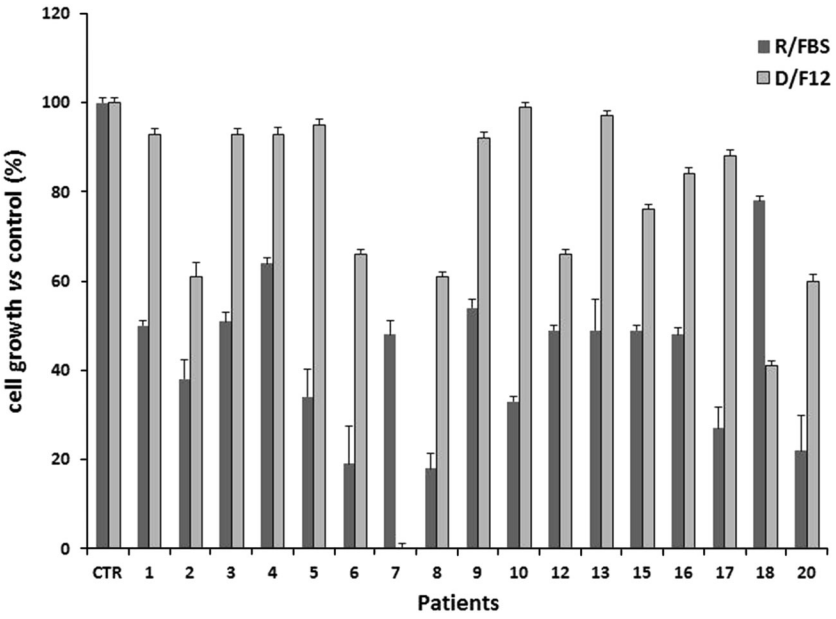

Fig. 4 Growth inhibition induced by CDDP (20 μM) in short-term primary cultures from NSCLC samples maintained in R/FBS or D/F12 culture conditions. Analysis could not be feasible in three patients (11, 14 and 19). (R/FBS vs. D/F12 $p < 0.001$) by Student's *t* test. The data represent the means of at least three independent experiments. *CTR* untreated control

($p < 0.0001$), in agreement with results obtained with the NSCLC cell lines. We then studied the relationship between the resistance to CDDP and the percentage of CD133- or EpCAM-positive cells in primary tumor D/F12 cultures (Suppl. Fig. 5). These data showed a trend toward an inverse correlation only between CDDP cytotoxicity and CD133. Unfortunately, the afatinib cytotoxic activity could not be assessed in these short-term primary cultures due to the limited number of cells available.

EGFR Activation in Non-Differentiated and Adherent NSCLC Cell Lines

In order to elucidate whether the increased sensitivity of the non-adherent spheres toward afatinib could be related to the increased EGFR activation, Western blot was carried out in NSCLC cell lines maintained in D/F12 or R/FBS conditions. A stronger expression of the phosphorylated form of EGFR (P-EGFR) was detected in all the cell lines maintained in D/F12, including the *EGFR-WT* A549 (Fig. 5a). To determine if growth factors contained in the culture medium might have been responsible for the increased EGFR activation, the same analyses were performed in the cell lines cultured in R/FBS or D/F12, starved from serum or EGF, respectively, for 24 h. As expected the A549 serum-starved cultures did not show P-EGFR while the serum-starved H1650 and H1975, harboring *EGFR*-activating mutations, retained EGFR phosphorylation. In addition, in starved cultures, P-EGFR levels were higher in D/F12 cells than in their R/FBS counterparts regardless of the *EGFR* mutational status, although the signal intensity was stronger in the H1650 and H1975 than in the A549 cells (Fig. 5b).

Altogether, these data suggest that the higher level of EGFR activation, observed in D/F12 cultures, might be responsible for the enhanced sensitivity to afatinib, particularly in the *EGFR*-mutated cell lines.

Discussion

In the present study, we investigated the sensitivity of CD133/EpCAM-positive NSCLC cells to CDDP or afatinib. To this end, NSCLC cell lines, as well as tumor cells derived from NSCLC surgical specimens, were grown in non-differentiating culture conditions to obtain enrichment in CSC-like, CD133/EpCAM-positive subpopulations (Chen et al. 2012).

The three NSCLC cell lines used in the current study were chosen for the absence or presence of activating mutations in the *EGFR* gene. NSCLC cell lines and tumor samples, cultured in D/F12 medium, grew in vitro as non-adherent spheres and showed an increased expression of CD133 and EpCAM. Of note, only about one-half of cells co-expressed the two markers, which identify only partially overlapping cell populations. Moreover, three out of four CD133/EpCAM-enriched primary cultures, derived from one squamous cell carcinoma and three adenocarcinomas, gave rise to tumor formation when transplanted subcutaneously into immune deficient NOD/SCID mice, indicating the tumorigenic potential of most of these cultures. CD133/EpCAM-enriched NSCLC lines also showed resistance to

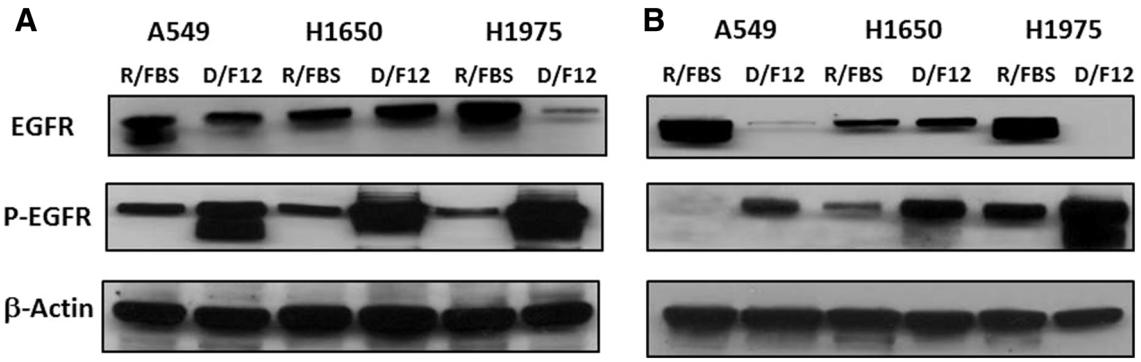

Fig. 5 Representative experiment ($n = 2$) of Western blot of NSCLC cell lines maintained in differentiating or non-differentiating cultured conditions. **a** Cells were cultured in R/FBS or D/F12. **b** Cells in R/FBS medium were deprived of serum, and cells in D/F12 medium were deprived of EGF for 24 h. β-Actin expression was assessed as loading control

CDDP treatment in vitro compared to cells maintained in standard culture conditions. Furthermore, the majority of primary cultures (82 %) grown in D/F12 demonstrated resistance to CDDP relative to their R/FBS counterparts. In addition, both *EGFR*-mutated and *WT* cell lines showed an increase in CD133-positive cells, after CDDP treatment in vitro. Altogether, these findings confirm previous reports showing that the conventional chemotherapy, though apparently efficient in reducing tumor burden, might spare drug-resistant cells with CSCs features (Bertolini et al. 2009; Levina et al. 2008).

The therapeutic management of NSCLC patients has been improved in the last years, due to the identification of driver mutations of specific oncogenes, and the development of new drugs targeting these oncogenic molecules or their downstream signaling pathways (Favoni and Alama 2013). In particular, the occurrence of specific mutations, which lead to constitutive activation of the EGFR, has allowed the identification of subgroups of patients potentially responsive to EGFR-TKIs (Ono and Kuwano 2006; Pao et al. 2004).

EGFR signaling is involved in various processes that contribute to cell proliferation, tumorigenesis and drug resistance (Zandi et al. 2007). Since the presence of CSC-like populations has been associated to the development of metastases, treatment resistance and recurrence, a relationship between EGFR activity and CSC features cannot be excluded (Peacock and Watkins 2008). Indeed, the impact of the EGFR pathway in CSCs was recently reported, as EGFR signaling is increased in the CSC-like populations of different types of tumors. EGFR-expressing glioma populations, with features of CSCs, showed the most malignant phenotype and were more sensitive to EGFR-targeted therapies (Mazzoleni et al. 2010). Moreover, aberrant activation of EGFR has been shown to promote the acquisition of CSC-like properties in head and neck squamous carcinoma cells, which displayed high sensitivity to gefitinib (Abhold et al. 2012). A crucial role of EGFR signaling has been also involved in the maintenance of NSCLC self-renewal (Singh et al. 2012) and a recent study reported that the stem cell-related marker CD133 was reduced upon treatment of H1975 spheroids with irreversible EGFR-TKIs (Galvani et al. 2013). Furthermore, CD133 was also identified as a predictive marker for long-term response to gefitinib in a fraction of NSCLC patients (Gottschling et al. 2012).

Of note, the present data show that *EGFR*-mutated NSCLC cells, enriched for CD133/EpCAM-positive subpopulations, display higher sensitivity to afatinib than differentiated cells. This finding may reflect the increased EGFR activation found in cells maintained in non-differentiating conditions relative to their differentiated counterparts. Indeed, the H1650 and H1975 cells cultured in D/F12 showed higher P-EGFR than R/FBS cells, even when starved from EGF. These findings suggest that EGFR activation of cells growing in D/F12 is not dependent on the presence of the exogenous EGF. Intriguingly, also the *EGFR-WT* A549 cells, cultured under D/F12 conditions, displayed increased P-EGFR. Therefore, it is conceivable that the activation of EGFR in NSCLC-*WT* cells might be related to the increased ability of the CD133-enriched cultures to self-produce EGFR-ligands. However, further experiments with a larger cell line panel, including both *WT* and mutated *EGFR*, are needed to confirm these data.

In conclusion, this study shows that both NSCLC cell lines and primary cultures, enriched for CD133-positive cells, were more resistant to CDDP but more sensitive to afatinib than the differentiated counterparts. It is conceivable that patients bearing *EGFR*-mutated NSCLC and expressing stem cell-related markers, such as CD133, may better benefit from further treatment with selected EGFR-TKIs, following the recurrence to standard regimens.

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Conflict of interest The authors declare no conflicts of interest regarding this study.

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