Published in final edited form as:

Exp Lung Res. 2014 October ; 40(8): 404–414. doi:10.3109/01902148.2014.938201.

Attenuation of lung cancer stem cell tumorigenesis and metastasis by cisplatin

Shenxu Wang^{#1,2}, Sai Ma^{#3}, Xiujuan Li^{#3}, Zengfu Xue⁴, Xiaotian Zhang³, Weiwei Fan³, Yongzhan Nie⁴, Kaichun Wu⁴, Xiaoyuan Chen⁵, and Feng Cao^{1,3}

¹Cardiology Department, Chinese PLA General Hospital, Beijing, China

²Cardiology Department, No. 150 Hospital of PLA, LuoYang, Henan, China

³Cardiology Department, Xijing Hospital, Xi'an, Shaanxi, China

⁴Digestive Disease Department, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China

⁵Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, Xiamen, China

These authors contributed equally to this work.

Abstract

Purpose—To investigate the effect of cisplatin on the growth and metastasis abilities of lung cancer stem cells (CSCs) via molecular imaging.

Materials and Methods—The expression changes of lung CSCs cell marker in A549-Luc-C8 human non-small-cell lung cancer (NSCLC) cell line with or without cisplatin treatment were detected by flow cytometry. The tumorigenesis and metastasis abilities of A549-Luc-C8 cells were monitored both in vitro and in vivo, and the mechanism was assessed by gene sequencing.

Results—About 1%–2% of CSCs were detected in A549-Luc-C8 cells and decreased CSCs percentage was observed after cisplatin treatment. Attenuated tumorigenesis and metastasis abilities of A549-Luc-C8 cells were found in cisplatin treated group.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at <http://www.tandfonline.com/page/terms-and-conditions>

Address correspondence to Feng Cao, Department of Cardiology, Chinese PLA General Hospital, 28# Fuxing Street, Beijing 100853, China. wind8828@gmail.com.

Publisher's Disclaimer: Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

Xiaozhi Bai and Dahai Hu in Department of Burns and Cutaneous Surgery of Fourth Military Medical University performed the qRT-PCR.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Conclusions—Decreased percentage of CSCs in A549-Luc-C8 cells can be induced by cisplatin treatment, which may partly be attributed to the attenuated expression of growth factors.

Keywords

cancer stem cells; cisplatin; growth factor; lung cancer; molecular imaging

INTRODUCTION

The survival rate of lung cancer has no obvious improvement in last 30 years [1], and chemotherapy based on cisplatin is only effective in about 30% of non-small-cell lung cancer (NSCLC) patients [2]. Cancer stem cells (CSCs) are believed to be intrinsically drug resistant, which may be one of the main reasons for tumor drug resistance and relapse [3, 4]. Chemotherapy may produce striking initial responses but does not maintain long-term remission if the rare CSCs are not well targeted and eliminated. The survived CSCs will lead to tumor regrowth, development as well as metastasis [5]. Thus, CSCs should be the main target for NSCLC therapy [6–8].

Researchers have found that CD44, CD24, ALDH1, and EpCAM could act as CSC markers in lung adenocarcinoma cell [9, 10], and Eramo et al. first suggested that CD133⁺ lung cancer cells maintained CSCs properties [11]. Levina et al. [12] and Bertolini et al. [13] have also proved that CD133⁺ lung CSCs can be enriched by transient or chronic cisplatin treatment in vitro. However, recent studies demonstrated that no enriched CSCs were detected in cisplatin-surviving breast cancer [14] or glioblastoma tumor cells [15] in vivo. Furthermore, Rahman et al. showed that targeting a putatively rare CSCs population had little effect on patient outcomes [16]. Though CSCs have been previously believed to be intrinsically more drug resistant as compared with the normal somatic stem cells [17], further studies are still needed to confirm the relationship between CSCs survival and drug resistance, which may be essential for personalized chemotherapy.

Growth factors play an important role in maintaining CSCs development both in vitro and in vivo [18–21]. Levina et al. have shown that growth factors such as VEGF and bFGF played an important role in CSCs-enriched tumors [12]. In this study, we aimed to investigate the effect of cisplatin on CSCs of A549-Luc-C8 human NSCLC cell line via molecular imaging and to reveal the underlying mechanism by RNA sequencing techniques.

MATERIALS AND METHODS

Cell Culture

The A549-Luc-C8 cell line was obtained from the Caliper Life Science Company (Caliper Life Science, Inc., CA, USA). A549 cells were stably transfected with North American Firefly Luciferase (Fluc) gene with CMV promoter as previously reported, named as A549-Luc-C8 cells [22]. A549-Luc-C8 cells were cultured in RPMI medium 1640 (Hyclone, USA), supplemented with 10% FBS (Hyclone, USA). 10 μ M cisplatin was used in our study as previously reported [23, 24]. In cisplatin treated group, cells were treated with 10 μ M cisplatin (Sigma-Aldrich, St. Louis, MO) for 72 hours. After that, cisplatin was removed and

cells were cultured for another 12 hours to obtain +Cis cells. A549-Luc-C8 cells without cisplatin treatment were collected as -Cis cells. Cells were visualized by light electron microscopy (Olympus BX51, Olympus, Tokyo, Japan).

Flow Cytometry Analysis

Side population (SP) analysis was performed as previously described [25]. For CD133 analysis, 1×10^6 -Cis or +Cis cells were trypsinized and resuspended in 300 μ L phosphate buffered saline (PBS) and incubated in the dark for 30 minutes with PE-CD133 or PE-IgG2b (both from eBioscience, San Diego, CA, USA). Dead cells were excluded by PI gating and samples were analyzed by FACS Aria III (BD Biosciences, San Jose, CA, USA).

Western Blot Analysis

Western blot analysis was performed as previously described [26]. Cells were washed by PBS for three times, scraped into a lysis buffer (Beyotime, Shanghai, China) with proteinase inhibitor (Beyotime, Shanghai, China). After centrifugation, the supernatant was collected. The concentration was determined using bicinchoninic acid protein assay (Beyotime, Shanghai, China). Equal amount of proteins was electrophoresed on a 6% or 8% Bis-Tris gel and transferred to nitrocellulose membrane (Beyotime, Shanghai, China). After being blocked by 5% skim milk in Tris-buffered saline, membranes were incubated with primary antibodies of rabbit polyclonal anti-CD133 (1:200, Abcam, USA), rabbit polyclonal anti-Notch1 (1:500, Abcam, USA), or rabbit polyclonal anti- β -actin (1:1000, Abcam, USA) overnight at 4°C, followed by incubation with corresponding secondary antibodies at 37°C for 1 hour. The blot signal was detected by ECL detection system (Amersham, USA).

Tumorsphere Formation

-Cis and +Cis cells were suspended in serum-free RPMI medium 1640 containing 2% B27 supplement (Invitrogen, San Diego, CA, USA), 20 ng/mL human recombinant basic fibroblast growth factor (bFGF-2, Peprotech, NJ, USA), and 20 ng/mL human epidermal growth factor (hEGF, Peprotech, NJ, USA). Then cells were planted in a 96-well ultra-low attachment plate (Corning Life Sciences, Acton, MA) at the density of 10–50 cells/cm². The tumorsphere formation was observed 2 weeks and 4 weeks later, respectively, and the number of tumorspheres was measured at the second week per $\times 200$ field by light electron microscopy (Olympus BX51, Olympus, Tokyo, Japan).

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described [26]. A549-Luc-C8 cells or tumorspheres were plated onto poly-L-ornithine-coated glass coverslips for 4 hours, fixed in 4% paraformaldehyde for 15 minutes at room temperature and then washed with PBS. Then, A549-Luc-C8 cells and spheres were incubated with OCT4 (1:200, Santa Cruz, CA, USA) overnight at 4°C, and the secondary antibody was goat anti-mouse FITC-IgG (Zhong Shan Company, Beijing, China) or goat anti-mouse cy3-IgG (Zhong Shan Company, Beijing, China). Cells were washed by PBS, stained with 4',6-diamidino-2-phenylindole (DAPI), and monitored by a FluoView FV1000 laser scanning confocal microscope (Olympus).

Animal Models

Nude mice were purchased from the Shanghai Laboratory Animal Center of China and maintained in the Animal Resources Center of the Fourth Military Medical University. The protocol was approved by the Committee on the ethics of Animal Experiments of the Fourth Military Medical University (permit number: 2010–09–012).

For tumorigenicity study, 5×10^3 , 5×10^4 , and 5×10^5 +Cis or –Cis cells suspended in 20 μ L PBS with 20 μ L matrigel (BD Bioscience, USA) were subcutaneously (s.c.) injected into the two points of the right or left flank of nude mice ($n = 2$ for each group). The tumor size was measured with a caliper every week, and the tumor volume (mm^3) was calculated at $[\text{length (mm)} \times \text{width (mm)}^2]/2$. Mice that were injected with 5×10^5 cells were sacrificed on the 25th day after cell injection when the tumor reached its maximum size about 300–500 mm^3 . Mice those were injected with 5×10^4 or 5×10^3 cells were sacrificed on the 25th or 86th day when the mice reached cachexia as previously reported, such as an excessive, involuntary loss of fat and lean tissue [27, 28]. In addition, 1×10^6 +Cis or –Cis cells were intravenously (i.v.) injected into the tail vein of nude mice ($n = 5$ for each group) to analyze the tumor cell metastasis ability. Mice were imaged by bioluminescence imaging (BLI) and sacrificed on the 80th day after cell injection. Lung, liver, kidneys, and lymph nodes were immediately removed to detect the metastatic nodules.

For therapy experiment, 1×10^6 +Cis or –Cis cells were s.c. injected into two points of the right or left flank of nude mice ($n = 8/\text{group}$). Nude mice were intraperitoneally (i.p.) injected with cisplatin (10 mg/kg) or PBS (100 μ L) twice a week for 3 weeks. Mice were imaged twice a week and sacrificed at the end of the third week.

Bioluminescence Imaging (BLI)

BLI was performed as previously described [29]. Briefly, 1×10^6 A549-Luc-C8 cells, lung, liver, kidney, and lymph nodes were dissociated and placed into a 24-well plate, incubated with 500 μ L PBS and 1 μ L D-luciferin (40 mg/mL, Caliper Life Science, Inc., CA), and then detected 1 minute later by the Xenogen IVIS Kinetics imaging system (Caliper Life Science, Inc., CA). For in vivo imaging, mice were anesthetized by isoflurane, i.p. injected with D-luciferin solution (125 mg/kg) and then imaged by the Xenogen IVIS Kinetics imaging system (Caliper Life Science, Inc., CA). Data were acquired and analyzed by IVIS Living Imaging (Caliper Life Science, Inc., CA) software packages.

H&E Staining

Tumors, lung, liver, kidneys, and lymph nodes were stained with H&E as previously described [30].

Digital Gene Expression Sequencing of RNA and Statistical Analysis

Total RNA of the –Cis and +Cis tumors was isolated using Trizol (Invitrogen, USA) and measured using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). A total of 20 μ g RNA of each sample was used for RNA sequencing. The RNA was first fragmented into small pieces and then cDNA libraries were prepared according to the manufacturer's instruction (Illumina Inc., USA) and purified by the QIAquick PCR Purification Kit

(Qiagen). The cDNA was linked to the illumine PE adapters, and a range of cDNA fragments (200 ± 25 bp) was excised from the gel for downstream enrichment. Polymerase chain reaction (PCR) was performed to amplify the cDNA library by using Gex PCR primers according to the manufacturer's protocol. Then, the cDNA library products were sequenced on the Illumina Cluster Station and Genome Analyzer (Illumina). Standardized transcripts per million clean tags were used to compare the expression level of genes between –Cis tumors and +Cis tumors. Log2 ratio was used to measure the fold change in expression (+Cis versus –Cis tumors). In addition, false discovery+rate (FDR) adjustment was performed to obtain adjusted *P*-values for all genes. Potentially novel mRNAs were identified after filtering out all annotated mRNA tags or those matching the mitochondrial genome.

Quantitative Real Time PCR (qRT-PCR)

qRT-PCR was performed to validate the RNA-Seq analysis as previously described [26]. All the primer sequences were designed by Primer Express software and listed in Table 1.

Statistical Analysis

Each experiment was performed and repeated three times. The results were presented as mean $\pm$ SEM. Student's *t*-test or One-way analysis of variance (ANOVA) was used for analysis by GraphPad PRISM 5.0 software (San Diego, CA) and *P* < .05 was considered as statistically significant.

RESULTS

Identification of the CSC Characteristics Derived from A549-Luc-C8 Cells in Vitro

We first monitored the effect of cisplatin on CSCs in A549-Luc-C8 cells in vitro. SP analysis, which is a standard method for isolating CSCs [22], showed that about 1.9% CSCs existed in A549-Luc-C8 cells (Figure 1a). Since CD133 was previously reported as a marker of lung CSCs [11, 13], we detected the percent of CD133⁺ cells in –Cis and +Cis cells. Flow cytometry analysis revealed that about 1.1% CD133⁺ cells existed in –Cis cells (Figure 1b). In contrast to our initial hypothesis that the CD133⁺ cells may be enriched by transient cisplatin treatment, slight decrease of CD133⁺ cell percentage was found in +Cis cells as compared with –Cis cells (Figure 1c). Western blot further confirmed expression levels of CD133, Notch1 and CXCR4 proteins in +Cis cells were reduced as compared with that in –Cis cells (***P* < .01, Figure 1d and e).

Tumorigenesis Ability of +Cis Cells and –Cis Cells in Vitro

The tumorigenesis ability of +Cis and –Cis cells in vitro was tested by tumorsphere experiment. Obvious colonies were formed in –Cis cells after being cultured in RPIM 1640 supplemented with 20 ng/mL hHGF and 20 ng/mL bFGF for 2 weeks, but significantly less colonies were found in +Cis cells under the same condition (***P* < .01, Figure 2a and b). After culture for 4 weeks, the –Cis colonies were also significantly larger than +Cis colonies (Figure 2a). The expression of OCT4, a marker of lung CSCs [31], was elevated in tumorspheres as compared with that in A549-Luc-C8 cells by immunofluorescence staining (Figure 2c).

Tumorigenesis Ability of +Cis Cells and –Cis Cells in Vivo

Then, the tumorigenesis abilities of +Cis and –Cis cells were detected in vivo by BLI. BLI showed that the photon flux density of a single A549-Luc-C8 cell in vitro was 22 photons/second (Figure 3a). The tumorigenesis rate of –Cis cells was one out of four in 5×10^3 group, two out of four in 5×10^4 group, and 100% in 5×10^5 group (Table 2). Whereas +Cis cells showed no tumor growth in 5×10^3 or 5×10^4 group, and two out of four in 5×10^5 group (Table 2). BLI also confirmed that no signal could be detected in +Cis cells in 5×10^3 group on day 86 or in 5×10^4 group on day 25. The Fluc signal of +Cis cells in 5×10^5 group was significantly weaker than –Cis cells on day 25 (Figure 3b and c). Furthermore, tumor volume in +Cis group was significantly smaller than that in –Cis group after 5×10^5 cells injection (Figure 3d and e). Similar poorly differentiated mucinous carcinoma was observed by H&E staining in the –Cis and +Cis tumors (Figure 3f).

Metastasis Ability of +Cis and –Cis Cells in Vivo

1×10^6 +Cis or –Cis cells were injected into the nude mice ($n = 5$ per group) via tail vein to detect tumor metastasis ability. Lung, liver, kidneys, and lymph nodes were isolated and then monitored by BLI on day 80 in the presence of D-luciferin. Obvious Fluc signal could be detected in the lung tissue in –Cis group while no signal was found in +Cis group (Figure 4a). Higher metastatic frequency to the lung was observed in –Cis group than in +Cis group as was revealed by H&E staining (Figure 4b). Additionally, the lung metastasis was histologically consistent with poorly differentiated pulmonary adenocarcinoma (Figure 4c). No metastasis in liver, kidney, or lymph node was detected by BLI or H&E staining (data not shown).

Drug Resistance Could not be Induced by Chronic Cisplatin Administration in Vivo

We also established an A549-Luc-C8 tumor mouse model with chronic cisplatin treatment both in vitro and in vivo. 1×10^6 +Cis or –Cis cells were injected into two points of the right or left flank of nude mice, and then the nude mice were i.p. injected with 10 mg/kg cisplatin ($n = 4$ per group) or 100 μ L PBS ($n = 4$ per group) twice a week for 3 weeks. ++Cis group showed attenuated Fluc signal in comparison with –Cis group (Figure 5a and b). Although with no statistical significance, the Fluc signal in –Cis group was a little bit higher than that in ++Cis or +Cis group. Decreased tumorigenic potential was also induced by cisplatin treatment especially in ++Cis group as compared with all the other groups. Cisplatin pretreatment in vitro (+Cis group) showed less tumorigenesis ability as compared with cisplatin pretreatment in vivo (–Cis group, Table 3). Minimum tumor volume was observed in ++Cis group as compared with the other groups (Figure 5c).

Decreased Growth Factors were Detected by RNA-Seq Technique

RNA-Seq technique was used to investigate the different gene expressions between –Cis and ++Cis tumors, with 154 genes upregulated and 163 genes downregulated in ++Cis tumors as compared with –Cis tumors (data not shown). The threshold of RNA-Seq used in determining the increased or decreased expression of growth factors is >1.5 fold change. But we also confirmed other vascular growth factors with a fold change <1.5 or <-1.5 for further research, such as SOX2, CD133, Notch 1, IGF2, VEGFC, PDGFA, BCRP3. The

CSCs markers, such as SOX2 [32], CD133 [11], Notch1 [33] as well as Musashi-1 [34] were downregulated in ++Cis tumors (Figure 6a). Vascular growth factors, such as VEGF-C, bFGF, IGF2, PDGFA, PDGFB, PDGFC, PDGFD and drug resistance molecule BCRP3 were also downregulated in ++Cis tumors as compared with --Cis tumors (Figure 6a). qRT-PCR results were consistent with the results of RNA-Seq (Figure 6b).

DISCUSSION

CSCs are previously believed to be intrinsically drug resistant [35–37]. However, Pajic et al. showed no intrinsic drug resistance ability of CSCs in breast cancer [14]. Thus, the drug resistant ability of CSCs still need more research. In the present study, by using molecular imaging and mRNA sequencing techniques, we demonstrated that transient cisplatin treatment can decrease the percentage of CSCs in A549-Luc-C8 lung cancer cells and, attenuate its tumorigenesis and metastasis abilities both in vitro and in vivo, which may be mediated partly by the decreased expression of growth factors.

We used a lung adenocarcinoma cell line model of A549-Luc-C8 cells, which was capable of monitoring the cell survival both in vitro and in vivo by BLI. Eramo et al. first showed that CD133 was the marker of lung CSCs [11], and further study indicated that CD133 is indicative for the drug-resistant phenotype of NSCLC [38]. By SP analysis as well as the flow cytometry for CD133 marker, about 1%–2% CSCs were confirmed in A549-Luc-C8 cells in the present study, which was consisted with previous studies [39, 40].

CSCs are believed to be the main reason of tumor metastasis and relapse [41]. Tumor-initiating capacity of CSCs has been the gold standard to verify the stemness of the selected cell [42], which was attenuated by transient cisplatin treatment both in vitro and in vivo in our study. No drug resistance phenomenon was found in our study by BLI in vivo, and this was in accordance with Huysentruyt's report [43]. After transient cisplatin treatment in vitro or chronic cisplatin treatment in vivo, the expression of CD133 in the cells was decreased in both groups. Meanwhile, the cells tumorigenesis and metastasis abilities were attenuated after cisplatin treatment.

Growth factors and their receptors can be targets for CSCs therapy [6, 44], and anti-angiogenic therapy may be effective for cancer therapy [45]. Levina et al. have detected that enriched growth factors can induce the tumorigenesis and metastasis abilities of CSCs [12]. The RNA sequencing result revealed decreased angiogenesis-related growth factors, such as bFGF, PDGFB, PDGFC, PDGFD in ++Cis tumors compared with --Cis tumors, which may account for the decreased tumorigenesis ability of lung CSCs [44]. In contrast to our results, Levina et al. found that lung CSCs were intrinsically resistant to transient cisplatin treatment in vitro in H460 lung cancer cells [12]. The discrepancy of data could be attributed to the fact that Levina et al. used SCID mice model in their study while we used nude mice. SCID mice are more immunodeficient than nude mice and may be much easier for tumor formation. Thus, the CSCs may have a complex role in chemotherapy and the intrinsic drug resistance ability may be dependent on the cell types and different tumor models.

The expression of BCRP is believed to be highly related with drug resistance. It was reported that BCRP was enriched in CSCs cells [46–48] and was able to transport anti-cancer drug out of cells [49]. Our data showed that BCRP was downregulated in our chronic cisplatin treatment models in vivo, which may also indicated that no drug resistant ability of A549-Luc-C8 cells was induced by cisplatin.

In summary, lung CSCs population can be decreased by cisplatin treatment, which may be due to decreased expression of growth factors. Thus, further studies are needed to detect the intrinsic drug resistance ability of CSCs and to choose proper chemotherapy regimen for clinical drug resistant patients.

Acknowledgments

This work was supported by the National Distinguished Young Scientists Grant of China (81325009) and National Nature Science Foundation of China (No. 81270168, No. 81090274), (FCao BWS12J037), Innovation Team Development Grant by China Department of Education (IRT1053), National Basic Research Program of China (2012CB518101).

REFERENCES

- [1]. MacKinnon AC, Kopatz J, Sethi T. The molecular and cellular biology of lung cancer: identifying novel therapeutic strategies. *Br Med Bull*. 2010; 95:47–61. [PubMed: 20643690]
- [2]. Socinski MA. Cytotoxic chemotherapy in advanced non-small cell lung cancer: a review of standard treatment paradigms. *Clin Cancer Res*. 2004; 10:4210s–4214s. [PubMed: 15217960]
- [3]. Adhikari AS, Agarwal N, Iwakuma T. Metastatic potential of tumor-initiating cells in solid tumors. *Front Biosci*. 2011; 16:1927–1938.
- [4]. Tomuleasa C, Kacso G, Soritau O, Susman S, Petrushev B, Aldea M, Buiga R, Irimie A. The fundamental mechanisms of metastatic spread and chemotherapy resistance in lung cancer. *Pneumologia*. 2011; 60:99–103. [PubMed: 21823362]
- [5]. Soltanian S, Matin MM. Cancer stem cells and cancer therapy. *Tumour Biol*. 2011; 32:425–440. [PubMed: 21318290]
- [6]. Gorelik E, Lokshin A, Levina V. Lung cancer stem cells as a target for therapy. *Anticancer Agents Med Chem*. 2010; 10:164–171. [PubMed: 20184538]
- [7]. Alison MR, Lebrenne AC, Islam S. Stem cells and lung cancer: future therapeutic targets? *Expert Opin Biol Ther*. 2009; 9:1127–1141. [PubMed: 19653862]
- [8]. Pine SR, Marshall B, Varticovski L. Lung cancer stem cells. *Dis Markers*. 2008; 24:257–266. [PubMed: 18525120]
- [9]. Okudela K, Woo T, Mitsui H, Tajiri M, Masuda M, Ohashi K. Expression of the potential cancer stem cell markers, CD133, CD44, ALDH1, and beta-catenin, in primary lung adenocarcinoma—their prognostic significance. *Pathol Int*. 2012; 62:792–801. [PubMed: 23252868]
- [10]. Karimi-Busheri F, Zadorozhny V, Li T, Lin H, Shawler DL, Fakhrai H. Pivotal role of CD38 biomarker in combination with CD24, EpCAM, and ALDH for identification of H460 derived lung cancer stem cells. *J Stem Cells*. 2011; 6:9–20. [PubMed: 22997842]
- [11]. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C, De Maria R. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ*. 2008; 15:504–514. [PubMed: 18049477]
- [12]. Levina V, Marrangoni AM, DeMarco R, Gorelik E, Lokshin AE. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties. *PLoS One*. 2008; 3:e3077. [PubMed: 18728788]
- [13]. Bertolini G, Roz L, Perego P, Tortoreto M, Fontanella E, Gatti L, Pratesi G, Fabbri A, Andriani F, Tinelli S, Roz E, Caserini R, Lo Vullo S, Camerini T, Mariani L, Delia D, Calabrò E, Pastorino U, Sozzi G. Highly tumorigenic lung cancer CD133+ cells display stem-like features

and are spared by cisplatin treatment. *Proc Natl Acad Sci USA*. 2009; 106:16281–16286. [PubMed: 19805294]

- [14]. Pajic M, Kersbergen A, van Diepen F, Pfauth A, Jonkers J, Borst P, Rottenberg S. Tumor-initiating cells are not enriched in cisplatin-surviving BRCA1; p53-deficient mammary tumor cells in vivo. *Cell Cycle*. 2010; 9:3780–3791. [PubMed: 20855963]
- [15]. Ding L, Yuan C, Wei F, Wang G, Zhang J, Bellail AC, Zhang Z, Olson JJ, Hao C. Cisplatin restores TRAIL apoptotic pathway in glioblastoma-derived stem cells through up-regulation of DR5 and down-regulation of c-FLIP. *Cancer Invest*. 2011; 29:511–520. [PubMed: 21877938]
- [16]. Rahman M, Deleyrolle L, Vedam-Mai V, Azari H, Abd-El-Barr M, Reynolds BA. The cancer stem cell hypothesis: failures and pitfalls. *Neurosurgery*. 2011; 68:531–545. discussion 545. [PubMed: 21135745]
- [17]. Delude C. Tumorigenesis: testing ground for cancer stem cells. *Nature*. 2011; 480:S43–S45. [PubMed: 22169801]
- [18]. van Klaveren RJ, van't Westeinde SC, de Hoop BJ, Hoogsteden HC. Stem cells and the natural history of lung cancer: implications for lung cancer screening. *Clin Cancer Res*. 2009; 15:2215–2218. [PubMed: 19293258]
- [19]. Sakashita H, Ieta K, Haraguchi N, Inoue Y, Yoshizawa Y, Mori M. Cancer stem cell. *Gan To Kagaku Ryoho*. 2007; 34:1721–1729. [PubMed: 18030004]
- [20]. Aonuma M, Saeki Y, Akimoto T, Nakayama Y, Hattori C, Yoshitake Y, Nishikawa K, Shibuya M, Tanaka NG. Vascular endothelial growth factor overproduced by tumour cells acts predominantly as a potent angiogenic factor contributing to malignant progression. *Int J Exp Pathol*. 1999; 80:271–281. [PubMed: 10607018]
- [21]. Hofer EL, La Russa V, Honegger AE, Bullorsky EO, Bordenave RH, Chasseing NA. Alteration on the expression of IL-1, PDGF, TGF-beta, EGF, and FGF receptors and c-Fos and c-Myc proteins in bone marrow mesenchymal stroma cells from advanced untreated lung and breast cancer patients. *Stem Cells Dev*. 2005; 14:587–594. [PubMed: 16305343]
- [22]. Jenkins DE, Oei Y, Hornig YS, Yu SF, Dusich J, Purchio T, Contag PR. Bioluminescent imaging (BLI) to improve and refine traditional murine of tumor growth and metastasis. *Clin Exp Metastas*. 2003; 20:733–744.
- [23]. Barr MP, Gray SG, Hoffmann AC, Hilger RA, Thomale J, O'Flaherty JD, Fennell DA, Richard D, O'Leary JJ, O'Byrne KJ. Generation and characterisation of cisplatin-resistant non-small cell lung cancer cell lines displaying a stem-like signature. *PLoS One*. 2013; 8(1):e54193. [PubMed: 23349823]
- [24]. Wang S, Li W, Xue Z, Narsinh K, Fan W, Li X, Bu Q, Wang F, Liang J, Wu K, Cao F. Molecular imaging of p53 signal pathway in lung cancer cell cycle arrest induced by cisplatin. *Mol Carcinogen*. 2013; 52:900–907.
- [25]. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. 1996; 183:1797–1806. [PubMed: 8666936]
- [26]. Xue Z, Yan H, Li J, Liang S, Cai X, Chen X, Wu Q, Gao L, Wu K, Nie Y, Fan D. Identification of cancer stem cells in vincristine preconditioned SGC7901 gastric cancer cell line. *J Cell Biochem*. 2012; 113:302–312. [PubMed: 21913215]
- [27]. Huysentruyt LC, Shelton LM, Seyfried TN. Influence of methotrexate and cisplatin on tumor progression and survival in the VM mouse model of systemic metastatic cancer. *Int J Cancer*. 2009; 126:65–72. [PubMed: 19536778]
- [28]. Choi E, Carruthers K, Zhang L, Thomas N, Battaglino RA, Morse LR, Widrick JJ. Concurrent muscle and bone deterioration in a murine model of cancer cachexia. *Physiol Rep*. 2013; 1(6):e00144. [PubMed: 24400146]
- [29]. Cao F, Lin S, Xie X, Patel M, Zhang X, Drukker M, Dylla SJ, Connolly AJ, Chen X, Weissman IL, Gambhir SS, Wu JC. In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. *Circulation*. 2006; 113:1005–1014. [PubMed: 16476845]
- [30]. Salem N, Kuang Y, Wang F, MacLennan GT, Lee Z. PET imaging of hepatocellular carcinoma with 2-deoxy-2[18F]fluoro-D-glucose, 6-deoxy-6[18F] fluoro-D-glucose, [1–11C]-acetate and [N-methyl-11C]-choline. *Q J Nucl Med Mol Imaging*. 2009; 53:144–156. [PubMed: 19039303]

- [31]. Chen YC, Hsu HS, Chen YW, Tsai TH, How CK, Wang CY, Hung SC, Chang YL, Tsai ML, Lee YY, Ku HH. Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133-positive cells. *PLoS One*. 2008; 3:e2637. [PubMed: 18612434]
- [32]. Glinisky GV. “Stemness” genomics law governs clinical behavior of human cancer: implications for decision making in disease management. *J Clin Oncol*. 2008; 26:2846–2853. [PubMed: 18539963]
- [33]. Elias S, Liang S, Chen Y, De Marco MA, Machek O, Skucha S, Miele L, Bocchetta M. Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. *Oncogene*. 2010; 29:2488–2498. [PubMed: 20154720]
- [34]. Moreira AL, Gonen M, Rekhtman N, Downey RJ. Progenitor stem cell marker expression by pulmonary carcinomas. *Mod Pathol*. 2010; 23:889–895. [PubMed: 20305619]
- [35]. Yamauchi Y, Kohyama T, Takizawa H, Kamitani S, Desaki M, Takami K, Kawasaki S, Kato J, Nagase T. Tumor necrosis factor-alpha enhances both epithelial-mesenchymal transition and cell contraction induced in A549 human alveolar epithelial cells by transforming growth factor-beta1. *Exp Lung Res*. 2010; 36:12–24. [PubMed: 20128678]
- [36]. Karlic H, Herrmann H, Schulenburg A, Grunt TW, Laffer S, Mirkina I, Hubmann R, Shehata M, Marian B, Selzer E, Pfeilstöcker M, Pittermann E, Jäger U, Pehamberger H, Zielinski C, Valent P. Tumor stem cell research - basis and challenge for diagnosis and therapy. *Wien Klin Wochenschr*. 2010; 122:423–436. [PubMed: 20645015]
- [37]. Baguley BC. Multidrug resistance in cancer. *Methods Mol Biol*. 2010; 596:1–14. [PubMed: 19949917]
- [38]. Salnikov AV, Gladkich J, Moldenhauer G, Volm M, Mattern J, Herr I. CD133 is indicative for a resistance phenotype but does not represent a prognostic marker for survival of non-small cell lung cancer patients. *Int J Cancer*. 2010; 126:950–958. [PubMed: 19676044]
- [39]. Hsu HS, Lin JH, Huang WC, Hsu TW, Su K, Chiou SH, Tsai YT, Hung SC. Chemoresistance of lung cancer stemlike cells depends on activation of Hsp27. *Cancer*. 2011; 117:1516–1528. [PubMed: 21425153]
- [40]. Meng X, Li M, Wang X, Wang Y, Ma D. Both CD133+ and CD133– subpopulations of A549 and H446 cells contain cancer-initiating cells. *Cancer Sci*. 2009; 100:1040–1046. [PubMed: 19385971]
- [41]. Visvader JE. Cells of origin in cancer. *Nature*. 2011; 469:314–322. [PubMed: 21248838]
- [42]. Visvader JE. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev*. 2009; 23:2563–2577. [PubMed: 19933147]
- [43]. Huysentruyt LC, Shelton LM, Seyfried TN. Influence of methotrexate and cisplatin on tumor progression and survival in the VM mouse model of systemic metastatic cancer. *Int J Cancer*. 2010; 126:65–72. [PubMed: 19536778]
- [44]. Mimeault M, Hauke R, Mehta PP, Batra SK. Recent advances in cancer stem/progenitor cell research: therapeutic implications for overcoming resistance to the most aggressive cancers. *J Cell Mol Med*. 2007; 11:981–1011. [PubMed: 17979879]
- [45]. Deonarain MP, Kousparou CA, Epenetos AA. Antibodies targeting cancer stem cells: a new paradigm in immunotherapy? *MAbs*. 2009; 1:12–25. [PubMed: 20046569]
- [46]. Wang Z, Ravula R, Cao M, Chow M, Huang Y. Transporter-mediated multidrug resistance and its modulation by Chinese medicines and other herbal products. *Curr Drug Discov Technol*. 2010; 7:54–66. [PubMed: 20156136]
- [47]. Natarajan K, Xie Y, Baer MR, Ross DD. Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance. *Biochem Pharmacol*. 2012; 83:1084–1103. [PubMed: 22248732]
- [48]. Seo DC, Sung JM, Cho HJ, Yi H, Seo KH, Choi IS, Kim DK, Kim JS, El-Aty Am A, Shin HC. Gene expression profiling of cancer stem cell in human lung adenocarcinoma A549 cells. *Mol Cancer*. 2007; 6:75–82. [PubMed: 18034892]
- [49]. Nakanishi T, Ross DD. Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression. *Chin J Cancer*. 2012; 31:73–99. [PubMed: 22098950]

FIGURE 1.

Decreased percentage of lung CSCs was induced by transient cisplatin treatment in vitro. (a) SP analysis showed that about 1.9% CSCs existed in A549-Luc-C8 cells. (b and c) About 1.1% of A549-Luc-C8 cells were CD133⁺ cells (**P* < .05). The percent of CD133⁺ cells were slightly decreased in +Cis group as compared with -Cis group. (d and e) Decreased expression of CD133, Notch1, and CXCR4 was examined in +Cis cells as compared to -Cis cells by Western blot (**P* < .01).

FIGURE 2.

Attenuated tumorigenesis ability of +Cis cells in vitro. (a) Obvious tumorspheres were formed in -Cis cells while only small cell aggregates were observed in +Cis cells at 2-week time point. Phase-contrast photographs showed larger tumorspheres in -Cis cells as compared with +Cis cells at 4-week time point ($\times 400$). (b) More colonies were formed in +Cis group as compared with -Cis group after being cultured for 2 weeks ($*P < .01$). (c) OCT4+ cells were more enriched in tumorsphere cells as compared with normal A549-Luc-C8 cells ($\times 400$).

FIGURE 3.

Decreased tumorigenesis ability of +Cis cells in vivo. (a) BLI revealed that the Fluc signal density of A549-Luc-C8 cells was 22 photons/second. (b) No Fluc signal was detected in the +Cis tumors in 5×10^3 and 5×10^4 cell injection group. BLI showed markedly less Fluc signal in +Cis tumors as compared with -Cis tumors when injected with 5×10^5 cells. (c) Significantly attenuated Fluc signal was detected in +Cis tumors as compared with -Cis tumors in 5×10^5 cells group (* $P < .05$). (d) Nude mice with 5×10^5 cells injection were sacrificed on day 25 and the volume of +Cis tumor was significantly smaller than that of the -Cis tumor. (e) The tumor volume with 5×10^5 cells injection were measured on day 11, 14, 17, 21, and 25, respectively, and smaller tumor volume was found in +Cis tumors than -Cis tumors (* $P < .05$, ** $P < .1$, *** $P < .001$). (f) Poorly differentiated pulmonary adenocarcinoma of both -Cis and +Cis tumors was revealed by H&E staining ($\times 100$).

FIGURE 4.

Evaluation of the metastasis abilities of +Cis and -Cis cells in vivo. (a) Obvious Fluc signal in the lung tissue was detected in -Cis group while no Fluc signal was detected in +Cis group. (b) More lung metastases were detected in -Cis group than in +Cis group ($**P < .01$). (c) H&E staining confirmed the lung metastasis of -Cis cells.

FIGURE 5.

The chronic effect of cisplatin in vivo. (a) 1×10^6 +Cis or –Cis cells were injected into the right or left flank of nude mice ($n = 8$) and the nude mice were i.p. injected with PBS ($n = 4$) or cisplatin (10 mg/kg, $n = 4$) twice a week for 3 weeks. (b) Fluc signal of each group was detected on day 14, 17, 21, and 24. (c) Tumor volumes of each group were measured on day 14, 17, 21, and 24.

FIGURE 6.

Decreased growth factors were detected in --Cis tumors. (a) RNA sequencing showed that decreased expression of CSC markers, growth factors as well as drug resistance molecule were induced by cisplatin. (b) The expression levels of genes were confirmed by qRT-PCR.

TABLE 1

The Primers Used for qRT-PCR

Gene name	5'-3'
BMI1 forward	5'-CACTCTACACAGTCACGGGACAAAG-3'
BMI1 reverse	5'-GTCTGGTGAAGGTCCATGAGACAA-3'
CD44 forward	5'-TCAAGCCACAGAGTGGAGCAG-3'
CD44 reverse	5'-CATTCCCAGTGATGTGTTTGGAG-3'
CD133 forward	5'-AGTGGCATCGTGCAAACCTG-3'
CD133 reverse	5'-CTCCGAATCCATTGACGATA-3'
MSI1 forward	5'-AGAAGCACACGTGGCTGTAGGA-3'
MSI1 reverse	5'-CAGGGCTCAGCTGGATCAGA-3'
NOTCH1 forward	5'-TCCTAGTACTGCAATTCGGGAAA-3'
NOTCH1 reverse	5'-CAGATCCATGGAAAGAACTCAAAG-3'
SOX2 forward	5'-AGGCATTATGACACCACCGAAGA-3'
SOX2 reverse	5'-GAATTGGCTAGGGCAATCAATCAC-3'
bFGF forward	5'-TCTTCAGTTCGTGTGTGGAGACAG-3'
bFGF reverse	5'-GGGTGCGCAATACATCTCCAG-3'
PDGFB forward	5'-GCACCGGAAATTCAAGCACAC-3'
PDGFB reverse	5'-CATTGGCCGTCCGAATCAG-3'
PDGFC forward	5'-TGGGAACTGTGCCTGTTGTCTC-3'
PDGFC reverse	5'-ACACCGGTCTTTGGTCTCAACTG-3'
PDGFD forward	5'-AGACCAACTGGGAATCTGTCACAA-3'
PDGFD reverse	5'-GTCCAGAGCATCCGAATCA-3'
BCRP3 forward	5'-ACCATCAGTACCTGGCCGAGAG-3'
BCRP3 reverse	5'-TTGGTGGTCAGATTCAGTGAGGTC-3'
GAPDH forward	5'-TCCTAGTACTGCAATTCGGGAAA-3'
GAPDH reverse	5'-CAGATCCATGGAAAGAACTCAAAG-3'

TABLE 2

Subcutaneous Tumors Grown in Nude Mice

No. of tumor cells inoculated	–Cis	+Cis
5×10^3	1/4	0/4
5×10^3	2/4	0/4
5×10^3	4/4	2/4

TABLE 3

Tumor Formation After Chronic Treatment of Cisplatin in Vivo

No. of tumor cells inoculated	--Cis ^a	+--Cis ^b	--+Cis ^c	++Cis ^d
1×10^6	8/8	4/8	6/8	2/8

^a --Cis: A549-Luc-C8 cells without cisplatin treatment.

^b +--Cis: A549-Luc-C8 cells with 10 μ M cisplatin treatment in vitro for 72 hours.

^c --+Cis: A549-Luc-C8 cells with chronic 10 mg/kg cisplatin treatment in vivo.

^d ++Cis: A549-Luc-C8 cells with both 10 μ M cisplatin treatment in vitro for 72 hours and chronic 10 mg/kg cisplatin treatment in vivo.