Introduction

Lung cancer is the most deadly of all cancers[1]. Chemotherapy routinely plays an important role in the treatment of advanced and relapsed lung cancer. Regardless of the treatment regimen employed, the 5-year survival rate for lung cancer is less than 15%. This poor prognosis is mostly due to the development of drug resistance by lung cancer cells during treatment and the likelihood of subsequent metastasis[2]. Most patients will develop local or distant recurrence and widespread metastases following treatment. It is, therefore, very important to identify mechanisms of drug resistance and metastasis in order to improve the clinical efficacy of chemotherapy.

Many researchers have focused on cancer stem cells (CSCs)[3-12]. While CSCs are thought to be responsible for tumor regeneration after chemotherapy, direct confirmation of lung CSCs has not been established. Levina et al found that drug treatment could enrich and maintain CSCs and surviving cells have high tumorigenic and metastatic abilities. They called the cells drug-surviving cells (DSCs)[1]. In our study, we have focused primarily on DSCs, rather than on CSCs, because they are closer to the clinical requirements of drug resistance and their existence can be confirmed during drug treatment. Research involving DSCs will play an important role in the future cure of lung cancer. Little attention has been paid to DSCs to date.
While some drug-resistant lung cancer cell lines have been established \textit{in vitro}\textsuperscript{[13–16]}, there have been no published reports of drug surviving cell lines established directly from human patients with multi-drug-resistant lung cancer.

In this report we describe the establishment and characterization of a human lung cancer drug surviving cell line Am1010, directly derived from muscle metastases of a human lung adenocarcinoma, which exhibited multi-drug-resistance to cisplatin, taxol, and gefitinib.

**Materials and methods**

**Patient clinical status and follow-up**

A southern Chinese female, 44 years old, was admitted to the hospital with an unproductive cough for 3 months. She was found to have an enlarged lymph node of the neck. A positron emission tomography/computed tomography (PET/CT) scan showed a 3×6.6 cm tumor in the left lung with metastases in the left supraclavicular and mediastinal lymph nodes. A bone scan showed a developed skeletal tumor located in the third lumbar vertebra. Tissue obtained from the left supraclavicular lymph node biopsy revealed a poorly differentiated adenocarcinoma resulting from lung cancer. The chemotherapy regime consisted of Docetaxel (75 mg/m	extsuperscript{2}) and Carboplatin (400 mg/m	extsuperscript{2}) on day 1, repeated every 4 weeks for four cycles. Radiotherapy was concurrently administered to the lung and cervical regions with 40 Gy (2 Gy/fraction) for 20 days during the first cycle of chemotherapy. A daily dose of gefitinib (250 mg) was added during the subsequent 3 cycles of chemotherapy.

Establishment and maintenance procedure

Fresh tissue was excised from the metastatic tumor in right upper arm. Both necrotic and normal tissue were discarded. The remainder of the tumor tissue was then minced into small pieces (approximately 1 mm) with scissors. The minced tissue was shaken with a mixture of 0.1 mg/mL of deoxyribonuclease type I, 1 mg/mL collagenase type IV, and 0.5 mg/mL of hyaluronidase type V (all from Sigma Chemical, St Louis, MO) in culture medium at 150 revolutions per minute in a water bath at 37 °C for 3 h. The resulting cell suspension was washed in PBS balanced salt solution, applied to a Ficoll-Hypaque gradient (LSM, Organon Teknika, Durham, NC) and then centrifuged (1000×g for 30 min). The interface was collected and washed three times with Hank’s balanced salt solution and suspended in culture medium in order to obtain the tumor cells. Fibroblast were removed using a cell scraper when they were observed in the cell cultures.

**Light and electron microscopy**

Tumors were obtained from the patient’s left supraclavicular lymph node and upper arm muscles as well as from experimental animals inoculated with the cultured cells. The tumors were excised and fixed in 10% neutral formalin solution and sectioned for histological studies.

Am1010 cultured cells were fixed with acetone at -20 °C and processed for histological preparations. Slide preparations of the cultured cells were stained by hematoxylin-eosin counterstaining for morphology.

Growth studies

To determine the inhibitory effects of different drugs on the growth of Am1010 cells, we used the tetrazolium dye assay of Mosmann (1983). Briefly, 100 μL aliquots of an exponentially growing cell suspension (5×10\textsuperscript{4} cells per mL) were seeded in 96-well microtiter plates and incubated for 24 h. Then, 100 μL aliquots of each drug at various concentrations were added. After exposure to each drug for 0, 24, 48, and 96 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution (5 mg/mL in PBS) was added to each well and the plates were incubated at 37 °C for an additional 3 h. After centrifugation of the plates at 800×g for 5 min, the medium was aspirated from each well as completely as possible and 200 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan. The optical density was measured at 490 nm using Delta-soft ELISA analysis on a Macintosh computer connected to a Bio-Tek Microplate Reader (EL-340, Bio Metal-lics, Princeton, NJ).

**Chromosome analysis**

Mitotic cells were obtained from colcemid-treated cultures (0.1 μg/mL overnight). The cells were treated with 0.75% KCL solution for 15 min and fixed with Carnoy’s solution for approximately 1 h. After fixing, the cells were spread on a glass slide by the air drying technique and stained with Giemsa’s solution. Karyotype analysis was performed by taking photographs of well-spread metaphase plates.

**Growth of the human lung cancer cell line Am1010 in nude mice**

In order to test the ability of the Am1010 cells to grow and form tumors \textit{in vivo}, the cells were injected into five nude mice as part of a “tumor-take” test. Nude mice were inoculated with suspensions of 1×10\textsuperscript{6} cultured Am1010 cells and carefully kept in a hygienic environment. The growth of the cells was checked every 3 days after inoculation.

**Expression profiling analysis and confirmation by RT-PCR**

Expression profiling of the Am1010 cell line was compared
with the P0318 lung cancer cell line. We established the P0318 cell line from another patient who exhibited an isolated lung adenocarcinoma tumor in the left upper lung with no metastases (as determined by PET/CT scan). This patient was never treated with drugs or radiation.

The protocol for cell harvesting was as follows: one day before harvest, the cells were re-fed with the original volume and concentration of medium. Attached cells were harvested at ~80% confluence, as assessed by phase microscopy of each flask. In pilot studies, samples of medium showed no appreciable change in pH between re-feeding and harvest, and no color change in the medium was observed in any of the flasks harvested. The elapsed time from the incubator to the stabilization of the preparation was <1 min. Total RNA was extracted using the Qiagen (Valencia, CA) RNeasy Midi Kit according to the manufacturer’s instructions. The RNA was then quantitated spectrophotometrically and aliquoted for storage at ~80 °C.

An aliquot of 2 µg of total RNA was used for a microarray analysis of 22000 human genes with 70-mer oligo probes using the adapt dual-color and dye-swap hybridization strategy. The microarray procedure was performed according to a previous protocol described by Patterson et al[17]. Arrays were scanned with a LuxScan 10K scanner and the resulting TIFF images were analyzed with LuxScan 3.0 software (both from CapitalBio Corp, Beijing, China). The dual color microarray data were normalized with a LOWESS function in the R statistical software package. Only genes with consistent differential expression (above 2-fold change) in both dye-swap microarrays were considered differentially expressed.

We utilized end-point semi-quantitative RT-PCR (reverse transcription polymerase chain reaction) to confirm the results of the microarray data analysis using the same RNAs as the microarray analysis. Briefly, 1 µg of total RNA was reverse transcribed to synthesize cDNA and 1 µL of cDNA product was used in the PCR reaction. GAPDH and ACTB acted as internal controls. The PCR protocol was as follows: initial denaturation for 5 min at 95 °C, followed by 25 cycles of amplification and denaturation for 30 s at 95 °C, annealing for 30 s at 56 °C, extension for 30 s at 72 °C, and extension for 5 min at 72 °C. Finally, 5 mL of PCR product was analyzed by agarose gel electrophoresis. Table 1 includes the sequences of the primers used in RT-PCR validation.

Statistical method
Statistical analyses of drug resistance differences between two cell lines were performed using a Student’s t-test. Two-sided P values of <0.05 were considered significant. All analyses were performed using SPSS for Windows Version 11.5 (SPSS Inc, Chicago, IL).

Results
Morphology and chromosome characteristics of cultured Am1010 cells
The Am1010 cells in the initial phases of the primary culture were attached to the surface of the culture vessels and exhibited no significant growth during the first 2 weeks. After the latent period, the culture medium became strongly acidified. The culture was found to contain a significantly increased number of cells and was subcultured. The subsequent cultures produced a dominant growth of epithelial-like cells. Over 80 successful sequential passages occurred as of December 2009. Microscopic observation of cultures throughout this period detected no sign of contamination by other cells (eg, fibroblasts). Cell morphology was examined with a phase contrast microscope and two shapes of cells were found. Cells were observed as either a short fusiform shape or a pleomorphic shape. The connections between neighboring cells was quite loose. Most of the cells were small and displayed a short fusiform shape. Approximately 5% percent of the cells displayed a large pleomorphic shape with prominent nuclei and little cytoplasm. The Am1010 cells were noted to form spontaneous colonies in suspension (Figure 1A).

Tumor cell pleomorphism can be more clearly resolved with electron microscopy. Using electron microscopy two shapes of cells were observed. Larger cells with a low electron density were attached to the surface of the culture vessels and exhibited no significant growth during the first 2 weeks. After the latent period, the culture medium became strongly acidified. The culture was found to contain a significantly increased number of cells and was subcultured. The subsequent cultures produced a dominant growth of epithelial-like cells. Over 80 successful sequential passages occurred as of December 2009. Microscopic observation of cultures throughout this period detected no sign of contamination by other cells (eg, fibroblasts). Cell morphology was examined with a phase contrast microscope and two shapes of cells were found. Cells were observed as either a short fusiform shape or a pleomorphic shape. The connections between neighboring cells was quite loose. Most of the cells were small and displayed a short fusiform shape. Approximately 5% percent of the cells displayed a large pleomorphic shape with prominent nuclei and little cytoplasm. The Am1010 cells were noted to form spontaneous colonies in suspension (Figure 1A).

Statistical analyses of drug resistance differences between two cell lines were performed using a Student’s t-test. Two-sided P values of <0.05 were considered significant. All analyses were performed using SPSS for Windows Version 11.5 (SPSS Inc, Chicago, IL).

Table 1. Sequences of the primers used in RT-PCR validation.

| Gene   | GenBank (No) | Forward primer (5′−3′) | Reverse primer (5′−3′) | Size (bp) |
|--------|--------------|------------------------|------------------------|-----------|
| TNC    | NM_002160    | GAGATGCCAAGACTGCGTACA  | GTTGACACGGTGACGTCTCT   | 182       |
| CCND1  | NM_053056    | AGAACACCGGCTCAAGCTAC   | CCGAGACCTCGACTGGC      | 204       |
| COL1A2 | NM_000089    | CTACCCAACTTTGCCTCTAG   | GTCCTTCCCACATCTTGG     | 229       |
| ITGA1  | NM_181501    | TGTCCTTTGAAATGAAATGC   | TCTTTTGGGTCACATCGGA    | 109       |
| RRAS2  | NM_012250    | GTGTGAGACTTTTACTTGCTGG | AGTGAATTCCAGACTCTATCG  | 116       |
| PDGFC  | NM_016205    | GTTCTTCTGATACGGGGTCTAG | CCGATTGTTTACGATTAGG    | 126       |
| SHC1   | NM_003029    | CTATGCTACCTACGGCAAGTC  | TATGTTGGGATTGCTACTG    | 183       |
| ICA1M  | NM_000201    | GACCCCCAACCCTTGTGATA   | AGTCTTTTGGCGGATAGA     | 266       |
| F11R   | NM_016946    | TCACTTTGTAATGCAAAGGCTG | CTAACCTCGGTTTCTCCTA    | 110       |
| CDL7N  | NM_001307    | ATGTTATAGCTCTTGGGTTG  | TCAAGGGGTCGAATGTC      | 215       |
| CDH1   | NM_004380    | GAGGATGATGAGGAGGGTCTG | GGGATTCTGCGGCTTGAGTA   | 114       |
| GAPDH  | NM_002046    | TTGTGCCCTAATGACCCCCCTT | CTCCACACGCTACTGGG      | 202       |
| ACTB   | NM_001110    | CATGTACGTTGCTATCCGCC   | CTTCCTAATGTACGGACCAT   | 250       |
density were observed to have 2 or 3 prominent nuclei and smaller cells with a high electron density were observed to have smaller nuclei than the larger cells (Figure 2A). The presence of numerous mitochondria, endoplasmic reticulum, and Golgi complexes were commonly observed in the large cells. Lysosomes were frequently observed in the small cells, but mitochondria, endoplasmic reticulum, and Golgi complexes were rare (Figure 2B and 2C).

Chromosome analysis of the cells was performed by observing 50 metaphase plates. Chromosome numbers varied widely across plates and ranged from 68 to 120 with a mode of 91. Most of the cells were in the hypertriploidy or hypertetraploidy range in chromosome number but contained no useful marker chromosome as evidence that cells were of female origin (Figure 3).

**Am1010 resistance to cisplatin, taxol, and gefitinib**

The resistance of Am1010 cells to cisplatin and taxol was compared with the resistance of P0318 cells. P0318 (Figure 1B) is a lung cancer cell line that we established from a patient with lung cancer resistant to cisplatin and taxol (Figure 4B). The patient exhibited an isolated lung adenocarcinoma tumor in the left upper lung with no metastases (as determined by PET/CT scan) and never received drug or radiation treatment. The P0318 cell line will be described in detail in a separate research paper. In the present paper, P0318 is used as non-DSC comparison to Am1010.

The sensitivity of Am1010, P0318 and classic lung cancer cell lines A549 and H460 to cisplatin, taxol, and gefitinib was examined by continuous exposure to a range of drug concentrations. Cell survival was monitored after 96 h by the MTT assay. Figure 4A depicts the survival curves of Am1010 cells treated with each drug and includes the average concentration required to inhibit cell growth by 50% (IC_{50}). Data were pooled from independent experiments.

The Am1010 cell doubling time without drug treatment was 42.395 h. The cisplatin IC_{50} was 4.299 µmol/L and >10 µmol/L for the Am1010 and P0318 cells, respectively. The lung cancer cell lines, A549 and NCI-H460, were used as control cells that served as a resistance and sensitive contrast, respectively. The cisplatin IC_{50} was >10 µmol/L and 0.89 µmol/L for the A549 and NCI-H460 cells, respectively. The taxol IC_{50} was...
that drug treatment could enrich and maintain a population of cells that survived the treatment and called these cells drug surviving cells (DSCs). They also found that DSCs had high tumorigenic and metastatic abilities[19]. Using a cellular model that is resistant to chemotherapy, such as a drug surviving cell line, will be helpful in the search for new methods to combat drug resistance. Little attention has been paid to DSCs to date and there have been no published reports of drug surviving cell lines established directly from human patients with multidrug-resistant lung cancer.

In this report we describe the establishment and molecular characterization of a human lung cancer drug surviving cell line, Am1010, derived directly from muscle metastases of a human lung adenocarcinoma patient with resistance to cisplatin, taxol, and gefitinib.

As a new cancer cell line, Am1010 possesses unique morphology, ultrastructural morphology, and chromosomal characteristics compared to other lung cancer cell lines. Two general shapes of cells are found in the cell line: fusiform small cells and pleomorphic large cells. The cells form spontaneous colonies of suspended cells and each cell type has distinct organelles. The different adhesion of the distinct cell types suggests that they may possess different metastatic abilities. The differences in cell organelles will also play important roles in anti-treatment and metastasis. Most lung cancer cell lines possess multiple karyotypic abnormalities[19]. The ploidy of the majority of the Am1010 cells analyzed was within the hypertriploid or hypopentaploid range. We did not find marker chromosomes in Am1010, but we did note that Am1010 cells have more chromosomes than most lung cell lines[20, 21]. Whereas some studies have revealed associations between several chromosome abnormalities as well as various clinical and pathological parameters in NSCLC (non-small-cell lung cancer)[22], the relationship between drug resistance and metastasis remains unclear.

The Am1010 cell line was derived from a metastatic tumor after 4 cycles of chemotherapy. Because the metastatic tumor occurred after treatment, it is likely to have consisted of DSCs. The cell line also demonstrated in vitro multi-drug-resistance to cisplatin, taxol, and gefitinib. A notable characteristic of Am1010 is the similarity in drug resistance of its cultured cells to that of the original patient’s lung cancer.

Drug resistance is related to tumor invasion and metastasis. Our knowledge of invasion and metastasis in lung carcinogenesis has expanded and it may lead to more specific targeted therapies and better prognosis in the future[23]. In this research, we have focused on focal adhesion and cell adhesion pathways because they are critical for intercellular adhesion and maintenance of both normal and malignant tissue architecture. Deregulation of genes involved in these pathways is associated with tumor invasion, metastasis, and unfavorable prognosis. Available data indicate that alterations to the proteins involved in the focal adhesion and cell adhesion molecule (CAM) pathways are early incidents in cancer development. The altered expression of one or more of the genes in these pathways is associated with extensive cancer invasion.

**Expression profiling analysis**

Expression profiles of Am1010 and P0318 cells were compared. The microarray data analysis found that 3122 genes were differentially expressed with more than a 2-fold change. The top 10 enriched pathways of the differentially expressed genes, cytokine-cytokine receptor interaction, cell adhesion, arginine and proline metabolism, cell communication, leukocyte transendothelial migration, focal adhesion, complement and coagulation cascades, tight junction, MAPK signaling pathway, and axon guidance, are included in Figure 5.

The focal adhesion and cell adhesion pathways were examined further because they are critical for intercellular adhesion and maintenance as well as for normal and malignant tissue architecture. Figure 5 summarizes 11 genes that were found to be differentially expressed and are known to be involved in the focal adhesion (TNC, CCND1, COL1A2, ITGA1, Rras2, PDGFC, and SHC1) and cell adhesion (ICAM1, F11R, CLDN7, and CDH1) pathways. The differential expression of these genes was confirmed with semiquantitative RT-PCR. TNC, CCND1, COL1A2, ITGA1, Rras2, PDGFC, SHC1, and ICAM-I were confirmed to be up-regulated and F11R, CLDN7, and CDH1 were confirmed to be down-regulated in the DSC Am1010 cell line compared with the non-DSC lung adenocarcinoma P0318 cell line.

**Ability of Am1010 to grow in nude mice**

All Am1010 transplantation experiments performed on nude mice were successful and all the animals developed tumors. Most of the tumors became apparent after 20 to 30 days and grew to an appreciable size within 45 or 60 days after transplantation. The structure and cellular morphology of the tumors produced in the experimental mice were very similar. Figure 1C depicts typical features of a tumor produced in a nude mouse. The structure and cellular morphology of the tumor are strikingly similar to those of the original tumor from the patient (Figure 1D). The results of histopathological observations suggest that the cultured cells are histologically similar to the original tumor cells.

**Discussion**

The poor prognosis of lung cancer is largely due to the fact that lung cancer cells acquire drug resistance before or during treatment and subsequent metastasis. Levina, et al found
and progression.

Adhesion pathway genes found to be differentially expressed in Am1010 compared with P0318 cells include: TNC, CCND1, COL1A2, ITGA1, RRAS2, PDGFC, SHC1 ICAM1, F11R, CLDN7, and CDH1. This pathway plays an important role in cancer metastasis. P0318 is a non-drug surviving cell line derived from a patient with an isolated lung tumor of the same pathology as Am1010 (adenocarcinoma) but with no metastasis. The patient was not treated with drugs or radiation but P0318 is resistant to cisplatin, taxol, and gefitinib.

Nimwegen et al found that focal adhesion kinase is essential in the early phase of metastasis formation and involves invasion and migration processes leading to colonization of the lung by MTLn3 breast tumor cells. They also found that inhibi-
bition of FAK during the first 5 days of metastasis formation dramatically reduced the number of experimental lung metastases[23]. Focal pathway genes found to be highly expressed in AM1010 compared to P0318 cells include: TNC, CCND1, COL1A2, ITGA1, RRAS2, PDGFC, and SHC1. Lin et al found that intercellular adhesion molecule-1 (ICAM-1) in the cell adhesion pathway was also involved in monocyte adherence to epithelial cells and cancer cell invasion. High expression of ICAM-1 in human lung cancer specimens is correlated to a high risk for advanced cancers (stages III and IV)[23]. ICAM-1 was also up-regulated in the Am1010 cell line compared with the P0318 cell line.

With the exception of ICAM-1, most genes in the cell adhesion pathway are tight junction-associated proteins and their down-regulation indicates the fence of preventing cancer cells invasion was weakened. F11R, junctional adhesion molecule 1 (JAM-A/F11R), is a tight junction-associated transmembrane protein that has been shown to participate in the regulation of epithelial barrier function[26]. CLDN7 is an important claudin gene. Claudins are tight junction proteins that are essential for the tight sealing of cellular sheets and, therefore, are involved in the control of paracellular ion flux and maintenance of tissue homeostasis. Some studies have shown that claudins are down-regulated in various cancers[27, 28]. F11R, CLDN7, and CDH1 were down-regulated in Am1010 suggesting that Am1010 cells encounter fewer obstacles when they begin the invasion process from the primary tumor. Taken together, our results indicate Am1010, the drug surviving cell line derived after drug treatment, is likely to have a higher metastatic ability than P0318, the non-drug surviving cell line that was never treated with drugs.

The significantly altered pathways of Am1010 indicate it will be a suitable cellular model for studying the mechanism of metastasis. Although many efforts have been made to explore the mechanisms involved in the development of lung cancer, the genetic events involved in the metastasis of lung cancer are still unclear. Am1010 will provide a means for better study of the mechanisms behind DSCs.

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Author contribution
Jian-xing HE and Nan-shan ZHONG designed research; Hui-ling LI, Si-ming XIE, Wei WANG, Jun HUANG, Dan-ping WEN, and Qiu-hua DENG performed research; Liang ZHANG, Cheng-jie CAI, and Dao-yuan WANG analyzed data; Hui-ling LI wrote the paper.

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