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Original Article

Establishment and characterization of primary lung cancer cell lines from Chinese population

Chao ZHENG[1, #], Yi-hua SUN[2, #], Xiao-lei YE[1], Hai-quan CHEN[2, *], Hong-bin JI[1, *]

[1]*Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China;* [2]*Department of Thoracic Surgery, Shanghai Cancer Center, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China*

Aim: To establish and characterize primary lung cancer cell lines from Chinese population.
Methods: Lung cancer specimens or pleural effusions were collected from Chinese lung cancer patients and cultured *in vitro* with ACL4 medium (for non-small cell lung carcinomas (NSCLC)) or HITES medium (for small cell lung carcinomas (SCLC)) supplemented with 5% FBS. All cell lines were maintained in culture for more than 25 passages. Most of these cell lines were further analyzed for oncogenic mutations, karyotype, cell growth kinetics, and tumorigenicity in nude mice.
Results: Eight primary cell lines from Chinese lung cancer patients were established and characterized, including seven NSCLC cell lines and one SCLC cell line. Five NSCLC cell lines were found to harbor epidermal growth factor receptor (EGFR) kinase domain mutations.
Conclusion: These well-characterized primary lung cancer cell lines from Chinese population provide a unique platform for future studies of the ethnic differences in lung cancer biology and drug response.

Keywords: lung cancer; primary cell lines; epidermal growth factor receptor; small cell lung carcinomas; non-small cell lung carcinomas

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Introduction

Lung cancer is one of the most common causes of cancer-related death worldwide[1, 2], with a five year survival rate of approximately 15%. In China, the situation is even worse. The vast majority of lung cancers are carcinomas derived from epithelial cells and are pathologically divided into two major types: non-small cell lung carcinomas (NSCLC) (~80%) and small cell lung carcinomas (SCLC) (~20%).

Lung cancer cell lines are crucial for translational and biomedical research. Lung cancer cells were successfully cultured approximately 25 years after the establishment of a human cervical carcinoma (HeLa) cell line[3, 4]. Since then, many attempts have been made to establish permanent lung cancer cell lines[5–10]. The emergence of lung cancer cell lines reached a peak after serum-free chemically defined media (for example, ACL4, C-based medium, and HITES) were introduced[11, 12]. Since then, more than 200 lung cancer cell lines have been successfully established. These permanent cell lines, derived from either primary or metastatic cancers, provide important experimental systems for studying the genetic changes and biology associated with tumor initiation and progression. As a pure cancer cell population, the global using of *in vitro* permanent cell lines over the past several decades has led to great achievements, including elucidating the molecular and translational biology of cancer and further drug screening[3, 13]. To date, more than 9000 citations, including several important biomedical discoveries, have resulted from the usage of these lines[3]. However, it is noteworthy that among all of the widely used cell lines, almost none have a Chinese genetic background.

Studies demonstrate that ethnic differences in genetic background are important in defining cancer biology as well as in drug toxicity[14–21]. For example, the epidermal growth factor receptor (EGFR) kinase domain mutations occur in approximately 10% of NSCLC patients in the Caucasian population[22], but occur in 30%–50% of NSCLC patients from East Asian populations[15, 17, 18, 21, 23]. Ethnic differences in the expression of allelic variants may produce altered pharmacokinetics and

# These authors contributed equally to this work.
* To whom correspondence should be addressed.
E-mail hqchen1@yahoo.com (Hai-quan CHEN);
hbji@sibs.ac.cn (Hong-bin JI)
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result in differential toxicity for the same anticancer treatments[19, 20]. Understanding the causes of ethnic differences in cytotoxic metabolism may help to improve cancer treatment in the clinic. From this standpoint, the establishment of lung cancer cell lines from Chinese genetic background is urgently needed.

We have worked to establish Chinese lung cancer cell lines. A total of eight primary Chinese lung cancer cell lines were successfully established, including seven NSCLC cell lines and one SCLC cell line. We characterized most of these cell lines for oncogenic mutations, growth kinetics, karyotype, and tumorigenicity in nude mice. These cell lines provide a very useful platform for studying the ethnic differences in cancer biology and drug response in the future.

Materials and methods

Collection of clinical specimens

All patients were from the Chinese population and underwent surgery for potentially curative resections. The lung cancer specimens or pleural effusions were collected with patient consent. Solid tumors were immediately immersed in ice-cold RPMI-1640 supplemented with P/S (1000 U/mL penicillin G and 1000 mg/L streptomycin), while pleural effusions were kept on ice. Samples were transported to the laboratory for primary cell culturing within one hour of collection.

Preparation of lung cancer cells from resected samples

Solid tumor specimens were rinsed twice with PBS supplemented with P/S and finely minced with scissors. Both necrotic tissue and apparently normal tissue were discarded. Tumor fragments were then immersed into ACL4 medium (for NSCLC)[24, 25] or HITES medium (for SCLC)[11, 12] supplemented with 5% fetal bovine serum (FBS) and P/S, and they were pipetted more than 50 times. The cell suspension was then transferred to a collagen-coated flask. The pleural effusions were subjected to red blood cell lysis and then to Ficoll gradient separation to remove lymphocytes. The interface was collected and washed three times with PBS plus P/S. Cells were suspended in ACL4 medium with 5% FBS and P/S and then cultured in a collagen-coated flask. The medium was changed every 3 d. A cell scraper was used to remove visible fibroblast growth whenever it occurred. Once the cells were confluent, they were digested with 0.05% EDTA-trypsin for passage. All established cell lines were maintained in culture for more than 25 passages.

Karyotype analysis

Cells were seeded and cultured for 70 h or until confluence. Colchicine was added to cells with a final concentration of 0.2 μg/mL. Cells were incubated for another 2 h and then digested using 0.05% EDTA-trypsin and resuspended in 0.075 mol/L KCl. After fixation in 3:1 methanol/glacial acetic acid, cell suspensions were dropped onto ice-cold slides, which were then stained by Giemsa for 8 min. Chromosome ploidy was estimated from at least 29 metaphases per cell line under a microscope.

EGFR and KRAS mutation detection

Detection of EGFR and KRAS mutations was previously described[18, 21]. Briefly, genomic DNA and RNA were extracted from Chinese lung cancer cell lines per standard protocol (RNeasy Mini Kit, and QIAamp DNA Mini Kit, Qiagen, Hilden, Germany). Total RNA was reverse transcribed into single-stranded cDNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, EU, USA) and sequenced. The cDNA regions of EGFR (exons 18–22) and KRAS (exons 2–3) were then PCR amplified and sequenced. All mutations found from the cDNA sequencing were further confirmed by genomic DNA sequencing.

Fluorescence *in situ* hybridization (FISH)

Cells were applied onto slides as described in Karyotype analyses. Slides were then incubated in 0.01% pepsin solution (pH 2.0) for 10 min at 37 °C and then fixed in 1% formaldehyde for another 10 min at room temperature. Vysis EGFR probe (Abbott Laboratories, Des Plaines, IL, USA) was applied to the targeted area, and slides were sealed with rubber cement. Slides were then incubated at 83 °C for 5 min, followed by another 16 h at 37 °C. Slides were washed with 0.4×SSC/0.3% NP-40 at 73 °C for 4 min and then 2×SSC/0.1% NP-40 at ambient temperature for 2 min. Chromatin was counterstained with 4′,6-diamidino-2-phenylindole. Fluorescence was analyzed in more than 50 cells per cell line for both EGFR and chromosome 7 centromere signals. We divided the EGFR copy number variation into 6 categories (disomy, low trisomy, high trisomy, low polysomy, high polysomy, gene amplification) as previously described[26].

Cell growth assay

Cells were seeded in 96-well plates with ACL4 plus 5% FBS (5000 cells per well for cell lines SH-437, SH-450; 7000 cells per well for cell lines SH-416, SH-289). Cell growth kinetics were assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay daily as previously described[27].

Soft agar assay

To assess soft agar colony formation, each of the cell lines (SH-224, SH-289, SH-416, SH-437, and SH-450. CRL-5803 serves as a control, which was obtained from ATCC) was suspended in a top layer of RPMI 1640 containing 10% FBS and 0.4% Select agar at 5000 cells per well in 6-well plates and plated on a bottom layer of RPMI-1640 containing 10% FBS and 1% Select agar. Cells were stained with 0.5 mL crystal violet for 1 h.

Tumor formation in nude mice

Cells (5×10^6 to 1×10^7) were mixed with matrigel and then transplanted subcutaneously in nude mice. The animals were killed after 2 weeks, and the tumors were fixed in formalin for hematoxylin-eosin staining and pathological analysis.

Results

Establishment of the primary Chinese lung cancer cell lines

From 2008 to 2010, we collected and used 179 fresh lung can-

cer specimens for the establishment of primary Chinese lung cancer cell lines. Eventually, eight cell lines were successfully established and maintained in cell culture for more than 25 passages. Some cell lines even exceed 40 passages. Among these cell lines, seven are NSCLC cell lines, including three from adenocarcinomas, one from squamous cell carcinoma, one from adeno-squamous carcinoma, one from large cell carcinoma and one from poorly differentiated carcinoma (Figure 1, Table 1). All NSCLC cell lines were cultured using ACL4 medium with 5% FBS. Three cell lines including SH-543, SH-450, and SH-437 can also be maintained in RPMI-1640 with 10% FBS. Because SCLC patients seldom go through surgery and very few tumor specimens are available, we have established only one SCLC cell line.

The clinical and pathologic details for all the patients from whom the cell lines were derived are summarized in Table 1. Most lines were established from primary solid tumors, with typical pathological images shown in Figure 1, except SH-289, which was derived from pleural effusions. All of the patients were from the Chinese population with an age range from 49 to 71 years old and with different stages of disease.

Morphological appearance of primary Chinese lung cancer cell lines

Five NSCLC cell lines, including SH-224, SH-289, SH-416, SH-437, and SH-450, grow in cell culture as an attached monolayer (Figure 2). Adherent cells were, in general, large, and they exhibited characteristic epithelioid 'cobble-stone' morphology and were occasionally multi-nucleated or vacuolated. SH-450 cells were slightly unique due to their unconstrained growth and foci formation even after they reach 100% confluence. Another two NSCLC cell lines, SH-405 and SH-543, grew in suspension like SCLC cell lines. This is not common because the majority of NSCLC cell lines are adherent cells. Only 5% (8 of 154) of NSCLC cell lines grow as suspension cells, and none has been established before in a Chinese genetic background. Typical suspension cell growth was seen in the SCLC cell line SH-498, which grows as either single cell or cell clusters.

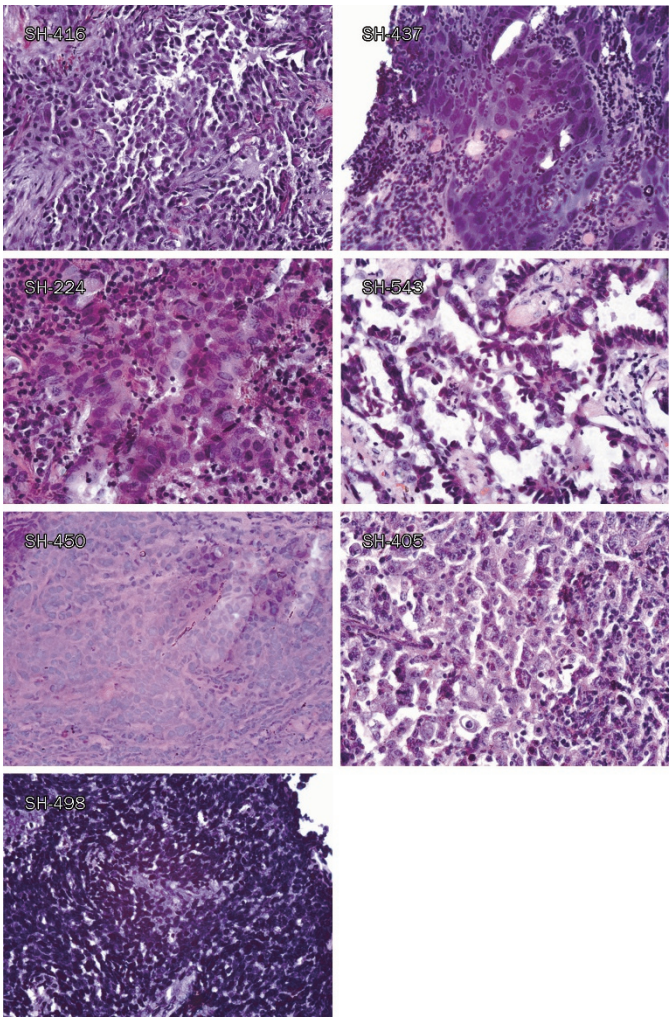


Figure 1. Typical pathological images of the tumor tissues used to establish primary Chinese lung cancer cell lines. Typical pathological images of the original tumors of primary Chinese lung cancer cell lines are shown, including SH-416 (SCC), SH-437 (LC), SH-224 (Ad), SH-543 (Ad), SH-450 (AS), SH-405 (PDE), SH-498 (SCLC).

Table 1. Clinical and pathologic summary of the established primary Chinese lung cancer cell lines.

Cell lines	Gender	Age (Year-old)	Pathology	Staging	Origin	EGFR mutations		EGFR CNV
						Tumor	Cell line	
SH-224	Female	71	Ad	III	Primary tumor	Exon 19 deletion	Exon 19 deletion	High polysomy
SH-289	Female	58	Ad	IV	Pleural fluid	Exon 19 deletion	Exon 19 deletion	Amplification
SH-543	Female	62	Ad	I	Primary tumor	WT	WT	Disomy
SH-416	Female	57	SCC	III	Primary tumor	Exon 19 deletion	Exon 19 deletion	Amplification
SH-450	Male	59	AS	III	Primary tumor	Exon 19 deletion	Exon 19 deletion	High polysomy
SH-437	Male	49	LC	II	Primary tumor	Exon 21 P848L	Exon 21 P848L	High polysomy
SH-405	Male	63	PDE	IIIa	Primary tumor	WT	WT	NA
SH-498	Male	53	SCLC	LS	Primary tumor	WT	WT	NA

Ad: Adenocarcinoma; SCC: Squamous cell carcinoma; LC: Large cell carcinoma; AS: Adeno-squamous carcinoma; PDE: Poorly differentiated carcinoma; WT: wild type; SCLC: Small cell lung carcinoma; LS: Limited stage; NA: not available; CNV: Copy number variation.

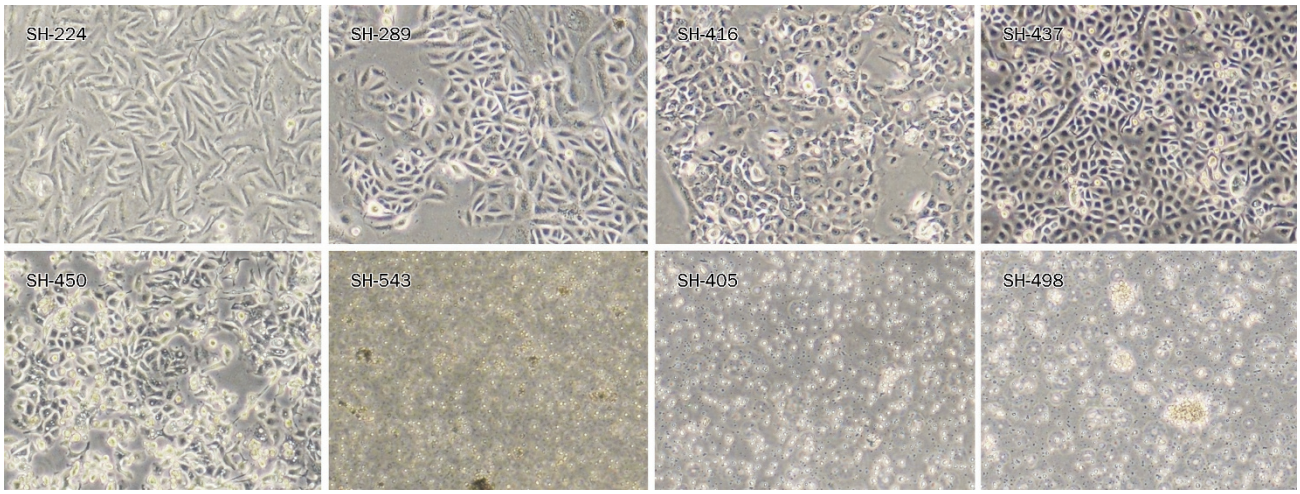


Figure 2. Representative morphology of primary Chinese lung cancer cell lines under light microscope. Typical morphology of cell lines is shown, including SH-224 (Ad), SH-289 (Ad), SH-416 (SCC), SH-437 (LC), SH-450 (AS), SH-543 (Ad), SH-405 (PDE), and SH-498 (SCLC).

Karyotype of the primary Chinese lung cancer cell lines

We have performed karyotype for the cell lines, except for SH-498 and SH-405, which grow very slow in suspension. The human origin of these cell lines is confirmed by a peak on nearly 46 chromosomes (Figure 3A). Most of the cell lines are aneuploid, consistent with their tumor origin. Significant subpopulations with both lower and higher chromosome ploidy were shown in all analyzed cell lines, with their modal chromosome numbers ranging from 36 to 107 (Figure 3B). SH-416 and SH-289 cells harbor a wide range of chromosomes number, while the suspension cell line SH-543 displayed almost uniformed diploidy (Figure 3A, 3C).

Status of EGFR mutations and gene amplification in the primary Chinese lung cancer cell lines

The EGFR kinase domain mutations, which are frequently associated with clinical tyrosine kinase inhibitor response, often occur in NSCLC patients from East Asia including China. We then asked if any of the above established cell lines harbor EGFR mutations, which may be useful for drug response studies. Using cDNA sequencing of the EGFR tyrosine kinase domain region, we found that five lines harbor EGFR mutations — four among them have exon 19 deletions (SH-224, SH-289, SH-416, and SH-450) and one has an exon 21 point mutation (SH-437) (Figure 4A). We further checked the gene amplification of EGFR allele, and the results from FISH analyses showed that all 5 cell lines displayed various degree of gene copy number increase (Table 1). Three cell lines (SH-224, SH-437, and SH-450) showed high polysomy, while another two (SH-289 and SH-416) showed gene amplification (Figure 4B).

Cell growth of the primary Chinese lung cancer cell lines in two dimension and soft agar

Five adherent NSCLC cell lines were used for further MTT assay and soft agar assay for testing their cell growth ability. Three cell lines with EGFR mutations SH-224, SH-437, and SH-450 grow faster than SH-289 and SH416 (Figure 5A).

Consistently, all the three cell lines (SH-224, SH-437, and SH-450) growing faster in two-dimension culture formed recognizable colonies in soft agar. In contrast, no colonies were seen in the two slow-growing cell lines SH-416 and SH-289 (Figure 5B).

Tumorigenicity of primary Chinese NSCLC cell lines

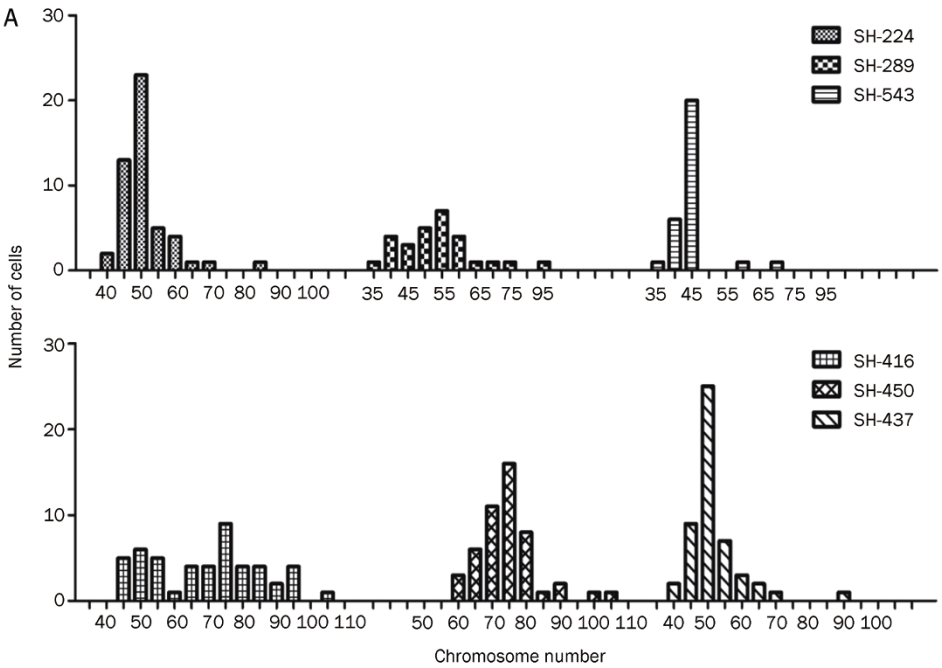
We further checked the tumor formation ability of these NSCLC cell lines in nude mice. Cell lines SH-224, SH-437, and SH-450 readily formed subcutaneous tumors in nude mice 2 weeks following transplantation. Surprisingly, SH-416, the cell line that failed to form colonies in soft agar, could also form a visible graft in nude mice at a low rate (1/4) (Figure 6A, 6B). This may somehow reflect the difference between *in vitro* and *in vivo* systems. Nonetheless, these data also suggest that SH-289 and SH-416 may have a low potential for tumor formation. The tumors grew locally as compact masses. In each instance the histological appearance of the tumors closely resembled the original tumors from which they were derived (Figure 1, 6C).

Discussion

The *in vitro* cell line model system has contributed significantly to cancer research and resulted in several seminal biomedical discoveries. However, the majority of these cancer cell lines are from Western populations, thus dramatically constraining the studies focusing on ethnic differences in cancer biology, drug response or toxicity between Chinese and Caucasian populations. In this study, we have established eight lung cancer cell lines from a Chinese genetic background. These well-characterized cell lines will provide an ideal platform for stimulating studies in the future.

In general, it is considered much more difficult to establish lung cancer cell lines in comparison with other types of epi-

A



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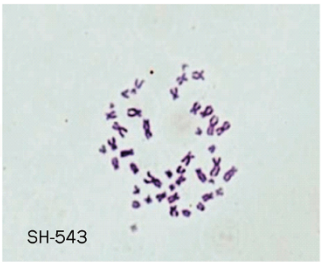
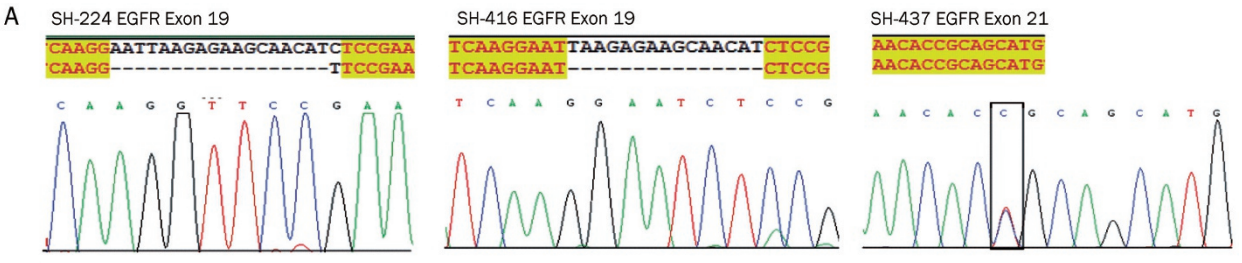


B

Cell line	Number of counted cells	Ploidy distribution	Mean
SH-224	50	40–84	50.9
SH-289	28	37–85	53.54
SH-543	29	36–72	45.31
SH-416	49	45–107	69.76
SH-450	49	58–107	74.39
SH-437	50	39–92	51.28

Figure 3. Ploidy and cytogenetic analyses of primary Chinese lung cancer cell lines. (A) Ploidy distribution of six Chinese NSCLC cell lines. (B) The summary of chromosome counting in the six Chinese NSCLC cell lines. (C) The karyotype of SH-543 is shown as one example.

A



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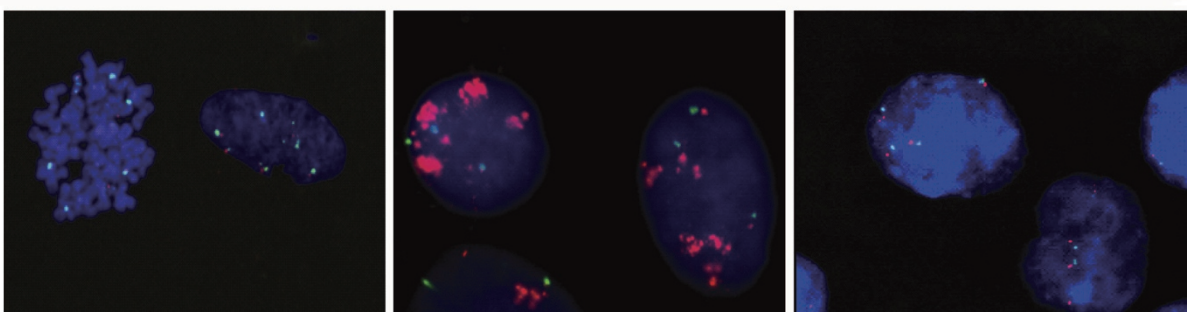

Figure 4 . Status of EGFR mutation and gene amplification in primary Chinese lung cancer cell lines. (A) DNA sequencing results showed EGFR mutations in the indicated cell lines. (B) FISH analyses showed either EGFR high polysomy or gene amplification in the indicated cell lines. Green: chromosome 7 centromere; Red: EGFR.

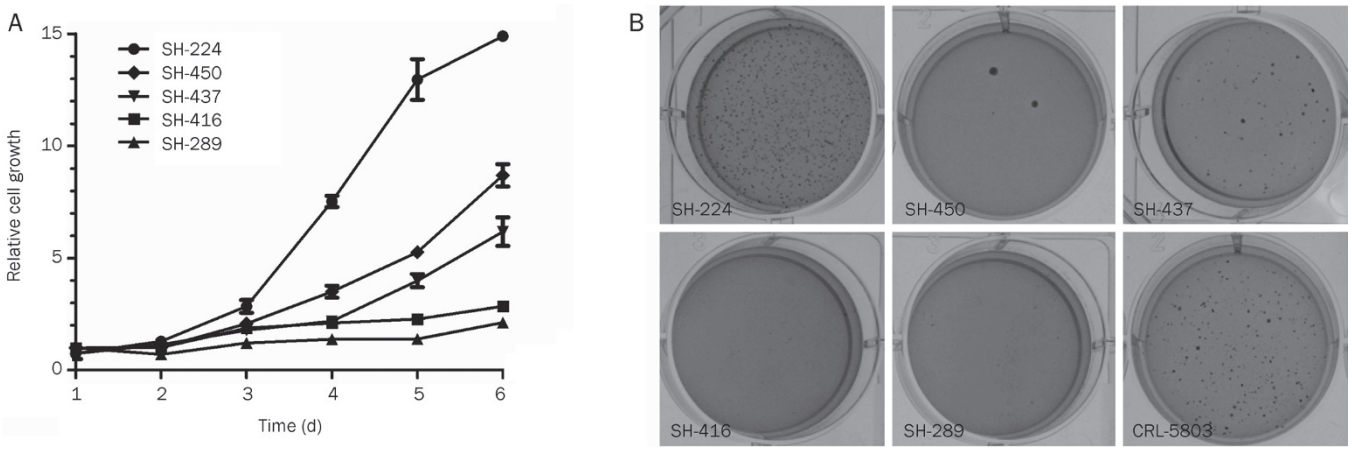

Figure 5. Cell growth of primary Chinese lung cancer cell lines in two dimension and soft agar. (A) The cell growth kinetics of the five adherent NSCLC cell lines are indicated. (B) The anchorage-independent cell growth of the five NSCLC cell lines. CRL-5803 serves as the control.

A

	Soft agar (Y/N)	Xenograft (tumor/total)
SH-224	Y	4/4
SH-450	Y	4/4
SH-437	Y	4/4
SH-416	N	1/4

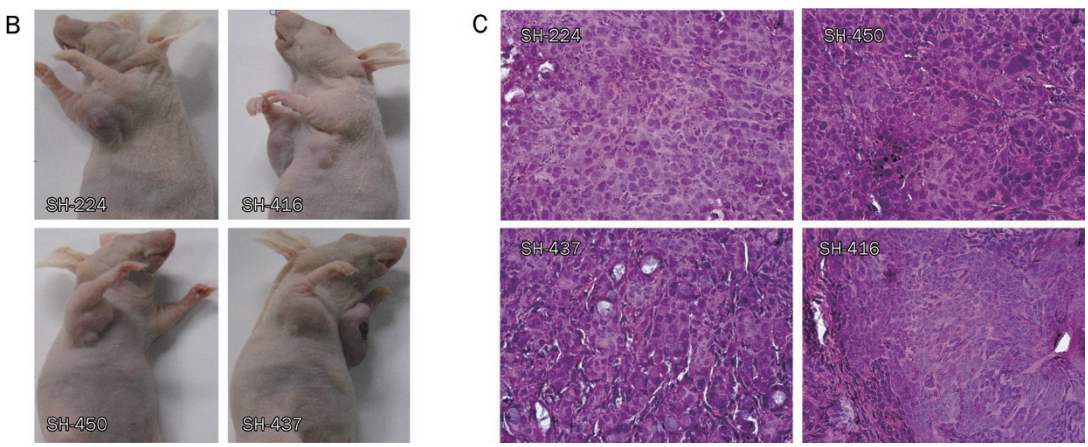

Figure 6. Tumor formation ability of primary Chinese NSCLC cell lines in nude mice. (A) The summary of tumorigenicity in four Chinese NSCLC cell lines in nude mice. (B) Representative photos are shown for the xenografts; (C) H&E staining of the tumors from xenografts.

thelia-originated cell lines[28]. Chemically defined media such as ACL3, ACL4, and HITES were first used for primary lung cancer cell culture with success. We tried here to add 5% FBS to the chemically defined medium ACL4, which stimulates the growth of not only the cancer cells but also the fibroblast. With great effort to eliminate fibroblast growth using scraping methods, we have successfully established eight Chinese lung cancer cell lines. It is possible that serum played an important role in making the floating cells attach to a culture dish. However, the success rate in our study still remains low (4.5%, Table 2). Further work is warranted to optimize the chemically defined medium.

To our surprise, five of the seven NSCLC cell lines we established harbor EGFR mutations as well as EGFR polysomy or allele amplification. EGFR mutations in all established Chinese NSCLC cell lines reach 71%. Through several decades of efforts, 21 lung cancer cell lines with EGFR kinase domain mutations have been developed worldwide: 12 with exon 19 deletions and 7 with the exon 21 mutation L858R and/or drug-resistant exon 20 mutation T790M (Table 3). Two uncommon mutations L861Q and G719S are also seen. Among these EGFR mutant cell lines, nine are from Caucasian populations,

Table 2. Summary of the success rate of all primary cell lines from different lung cancer subtypes.

Pathology	Success	Total	Success rate
Ad	3	83	3.6%
SCC	1	43	2.3%
AS	1	6	16.7%
LC	1	4	25%
PDE	1	11	9.1%
SCLC	1	4	25%
Others	0	28	0
Summary	8	179	4.5%

Table 3. Previously established lung cancer cell lines with EGFR kinase domain mutations.

Cell line	Pathology	EGFR mutation status	References
H1650	Ad	Exon 19 deletion (746–750)	[29]
HCC827	Ad	Exon 19 deletion (746–750)	[30]
HKULC 3	Ad	Exon 19 deletion (756–760)	[31]
PC3	Ad	Exon 19 deletion (747–749), A750P	[32]
PC9	Ad	Exon 19 deletion (746–750)	[33]
PC-14	Ad	Exon 19 deletion (746–750)	[32]
HCC2935	Ad	Exon 19 deletion (746–751), S752I	[34]
HCC4006	Ad	Exon 19 deletion (747–749), A750P	[35]
H820	Ad	Exon 19 deletion (746–751), T790M	[36]
HCC2279	Ad	Exon 19 deletion (746–750)	[37]
RERF-LC-Ad2	Ad	Exon 19 deletion (747–749), A750P	[32]
H1975	Ad	L858R and T790M	[29, 38]
H3255	Ad	L858R	[39]
HCC4011	Ad	L858R	[34]
11-18	Ad	L858R	[32]
LCSC#1*	Ad	L858R	[32]
LC2/Ad	Ad	L858R	[32]
KTA-7	Ad	L861Q	[32]
PC-7	Ad	G719S	[32]
RERF-LC-A1	SCC	L858R	[32]
KTSq-1	SCC	Exon 19 deletion (746–750)	[32]

eleven from Japan and one from Hong Kong. Here, we have established a comparable number of NSCLC cell lines with EGFR mutations in a Chinese genetic background. It is noteworthy that the majority of the previously established EGFR mutant cell lines are from lung adenocarcinomas, while two are from squamous cell carcinomas, which are not commonly used. In contrast, our collection of cell lines is composed of various pathologies including adenocarcinoma (3), squamous cell carcinoma (1), adeno-squamous cell carcinoma (1) and large cell carcinoma (1) (Table 1). In addition, SH-437 harbors an exon 21 point mutation P848L, which has not been previously reported in any cell line. Taken together, our effort to establish Chinese lung cancer cell lines was a very successful small step toward our long-term goal of establishing more than 30 Chinese lung cancer cell lines. These cell lines will provide important tools for studying the ethnic differences in lung cancer biology as well as in drug treatment between Chinese and Caucasian populations in the future.

Acknowledgements

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Author contribution

Hong-bin JI and Hai-quan CHEN designed the research; Chao ZHENG, Yi-hua SUN, and Xiao-lei YE performed the research; Hong-bin JI and Chao ZHENG wrote the paper.

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