

Original Article

Establishment and identification of human primary lung cancer cell culture *in vitro*

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Abstract: Objective: To explore a simple and practical method for human primary lung cancer cells culture *in vitro*. Methods: Tumor specimens from 6 lung cancer patients were isolated with collagenase digestion cultured *in vitro*. Then the characteristics of these cells were analyzed and identified by optical microscope observation, hematoxylin-eosin staining, immunocytochemistry, immunohistochemistry and tumor nude mice inoculation experiments, respectively. Results: Except for the small cell lung cancer, the other 5 samples were successfully isolated and cultured. The cultured cells showed typical characteristics of malignant cells and positive for cytokeratin 7 and 19. Moreover, the cancer cells readily formed subcutaneous tumors in nude mice and the pathological images of the transplanted tumor were consistent with its tumor origin. Conclusion: The primary culture for human lung cancer cells can be successfully achieved with the method of collagenase digestion.

Keywords: Lung cancer, primary cell culture, morphology, H-E staining, immunohistochemistry

Introduction

Lung cancer, as a common disease of the respiratory system, has accounted for the leading cause of cancer related death. In China, the registered lung cancer mortality rate increased by 464.84% in the past 3 decades, and about 600,000 people die of this disease every year, suggesting a high degree of malignance of this tumor [1, 2]. The pathogenesis of metastasis depends on both the host response and the intrinsic properties of the tumor cells [3-5]. Even in a same patient, the neoplasm displays a biological heterogeneity because of the co-existence of multiple cell populations with different invasive and metastatic abilities [3-5]. Therefore, each cancer patient needs to be regarded as an independent individual with a unique disease. To achieve effective personalized strategies in clinical practice, it is important to illustrate the biological behavioral characteristics of individual lung cancer cells

Since the advancement in *in vitro* culture technology, a number of lung cancer cell lines have been successfully identified and established,

which leads to our better understanding of tumor biological behavior. However, cell lines, as pure and immortalized cancer cell population, cannot truly represent the heterogeneous cancer *in vivo* after a long-term *in vitro* culture and repeated passage numbers, especially the origins of most of these cancer lines lack Chinese genetic background [3, 5, 6]. Thus, the development of primary tumor cell culture technology is of practical value for both the illumination of biological characteristics of individual lung cancer cells and transformation from bench research to bed side application.

In present study, human primary lung cancer cells was isolated with collagenase digestion, then the characteristics of cells were identified with morphology observation, hematoxylin-eosin (HE) staining, immunocytochemistry and tumor formation in nude mice, respectively. Through the establishment of a method for primary lung cancer culture *in vitro*, we hope to provide a model for the research of biological characteristics of lung cancer cells from individual patients and drug sensitive test for personalized therapy in clinical practice.

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Table 1. The basic information of the patients involved

Patients' No.	Gender	Age (year)	Pathological Types	Lesion Location
1	male	67	ASC	left upper lobe
2	male	47	SC	right lower lobe
3	female	56	AC	right upper lobe
4	female	51	SCLC	right lower lobe
5	male	56	SC	right upper lobe
6	male	72	AC	left upper lobe

Observation of cell growth

The growth state of live cells was observed under inverted microscope, and the morphology of cells was recorded on day (D) 1, 3, 5 and 7, respectively.

Hematoxylin-eosin staining

The digested cells at a density of 5×10^5 cells/mL were seeded on 6-well plates pre-placed with coverslips. At confluence of 80%, the cells were fixed in 4% polyformalin for 30 min and washed with phosphate buffered saline (PBS) for three times. After added with hematoxylin for 3 min, the fixed cells were underwent color separation with alcohol of 0.5% hydrochloric acid. Then, the cells were subsequently dyed with eosin, dehydrated with gradient ethanol, soaked with xylene and mounted with neutral balsam.

Immunocytochemistry

According to our previous method, the primary cultured lung cancer cells were seeded on 6-well plates pre-placed with coverslips at a density of 3×10^5 /ml [7, 8]. At 70-80% confluence, the cells were fixed with 4% formalin for 30 min at room temperature, then permeabilized for 15 min in 0.1% Triton X-100-PBS and treated with 0.3% H_2O_2 to inactivate endogenous peroxidase activity. After treated with blocking solution for 15 min, the cells were incubated with rabbit anti-human CK7 monoclonal or CK19 polyclonal antibody overnight at 4°C. A streptavidin-biotin detection reagent kit (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) with 3, 3'-diaminobenzidine tetrahydrochloride was used for signal detection and Harris hematoxylin was used as a counterstain. Vimentin and rabbit isotype IgG (20 µg/mL) (Sino-America, Shanghai, China) were used as negative control. The experiments were repeated three times.

Tumor inoculation in nude mice

The cells generated from one of the lung AC (2×10^7) were transplanted subcutaneously into the BALB/c nude mice (Hunan SLRC JingDa Laboratory Animal Co., Ltd). The animals were killed after 3 weeks to observe the tumor for-

Materials and methods

Sample collection

Surgical specimens (6 cases) were obtained from patients who underwent pulmonary lobe resection or pneumonectomy at the Thoracic Oncology Department, Zhongnan Hospital of Wuhan University in 2012. None of them received preoperative chemotherapy or radiotherapy. Of the six samples, four were male and two were female. The pathological types were adenocarcinomas (AC) of two, squamous cell carcinomas (SC) of two, adenosquamous carcinomas (ASC) of one and small cell lung cancer (SCLC) of one. Details were in **Table 1**. The study was approved by the Human Research Ethics Committee of Zhongnan Hospital, Wuhan University, and all participants completed an informed consent for the collection of tissue samples.

Primary cancer cell isolation and culture

The resected lung cancer tissues in a size of 1.0 cm³ without necrosis were immediately put into ice-cold RPMI-1640 supplemented with 100 U/mL penicillin G and 100 µg/mL streptomycin, and transported to the lab within 10 min. After the removal of blood clots, the samples were rinsed with sterile phosphate-buffered saline (PBS) twice and cut into small fragments in a size of about 1 mm³. Then, the fragments were incubated with collagenase of 1% (Sigma) in a gently shaking water bath for 1 h at 37°C. After passed through a 38 µm mesh sieve, the resulting cell suspension was washed twice and centrifugated at a speed of 300 g × 10 min. Then the pellet was diluted to 5×10^5 cells/mL and incubated in a culture bottle supplemented with Roswell Park Memorial Institute (RPMI) 1640 containing 10% heat-inactivated FBS (Gibco, Grand Island, NY, USA), in 37°C with 5% CO₂. After culture of 24 hrs, the first medium change was performed.

Human primary lung cancer cell culture

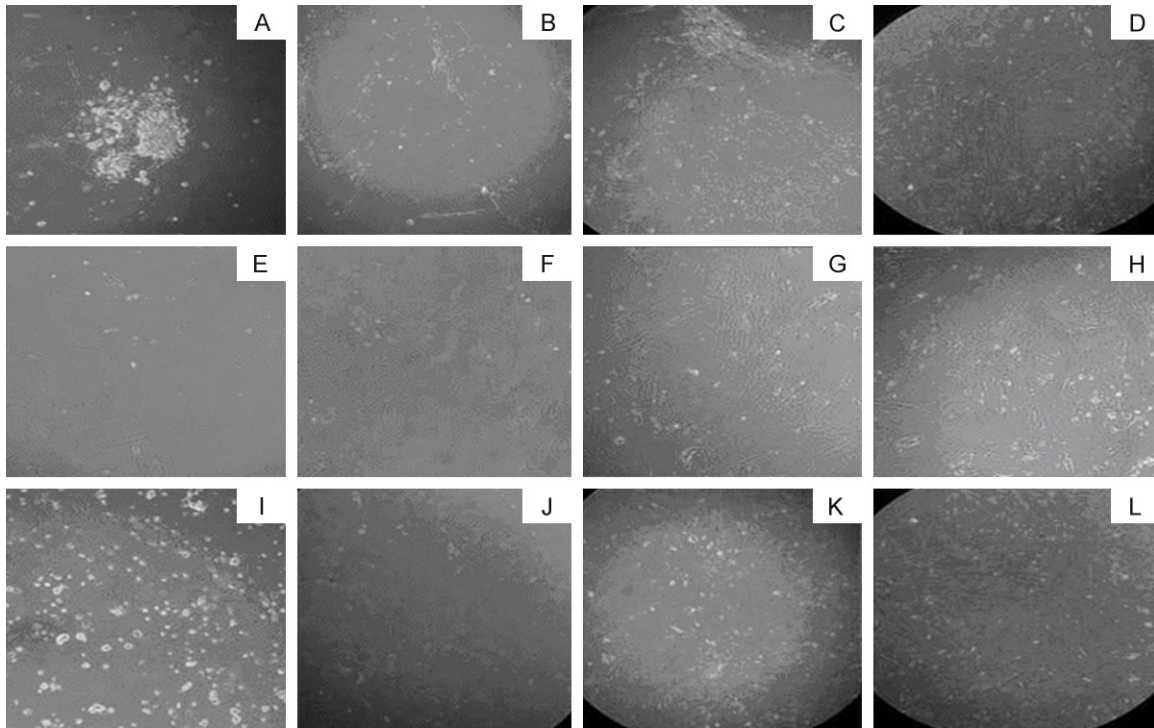


Figure 1. Growth state of primary cultured lung cancer cells *in vitro*. The growth state of primary cultured lung cancer cells were observed under the inverted microscope. A-D: The pictures of *in vitro* cultured cancer cells derived from squamous carcinoma patients on day 1, 3, 5 and 7, respectively; E-H: The pictures of *in vitro* cultured cancer cells derived from adenocarcinoma patients on day 1, 3, 5 and 7, respectively; I-L: The pictures of *in vitro* cultured cancer cells derived from adenosquamous carcinoma patients on day 1, 3, 5 and 7, respectively.

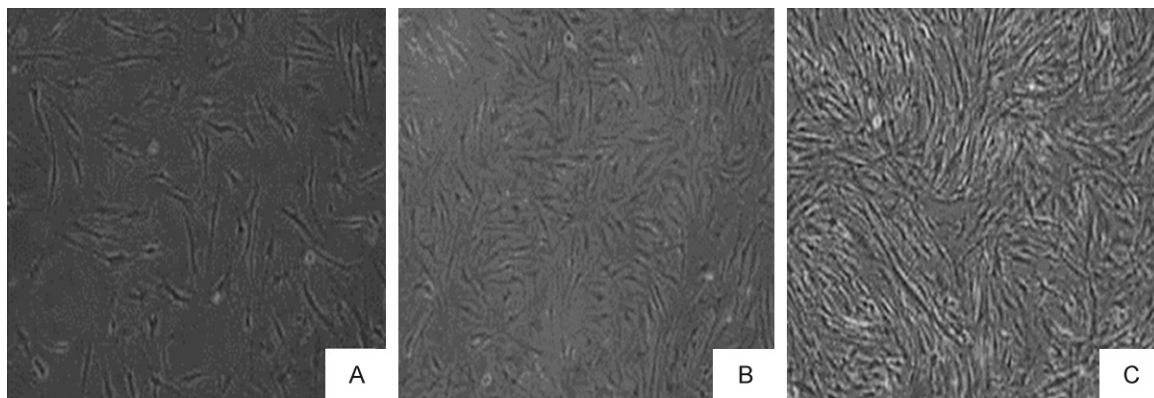


Figure 2. Morphology of primary lung cancer cells under inverted light microscope. The morphology of primary cultured lung cancer cells were observed and photographed under the inverted microscope. (100 \times).

mation and make hematoxylin-eosin (HE) staining section for pathological analysis.

Results

Growth state of primary cultured human lung cancer cells in vitro

Of the 6 cases, 5 samples were successfully isolated and cultured. The cells generated from

SCLC sample failed to survive *in vitro* because of the limited cell number. On the following day of isolation, a small quantity of adherent cells could be easily observed. As the culture time prolonged, the number of attached cells increased and the cells, appearing fusiform or polygon, gradually gathered into cluster or scattered over the bottom of the bottle. After 4-5 days, they entered into the rapid growth period

Human primary lung cancer cell culture

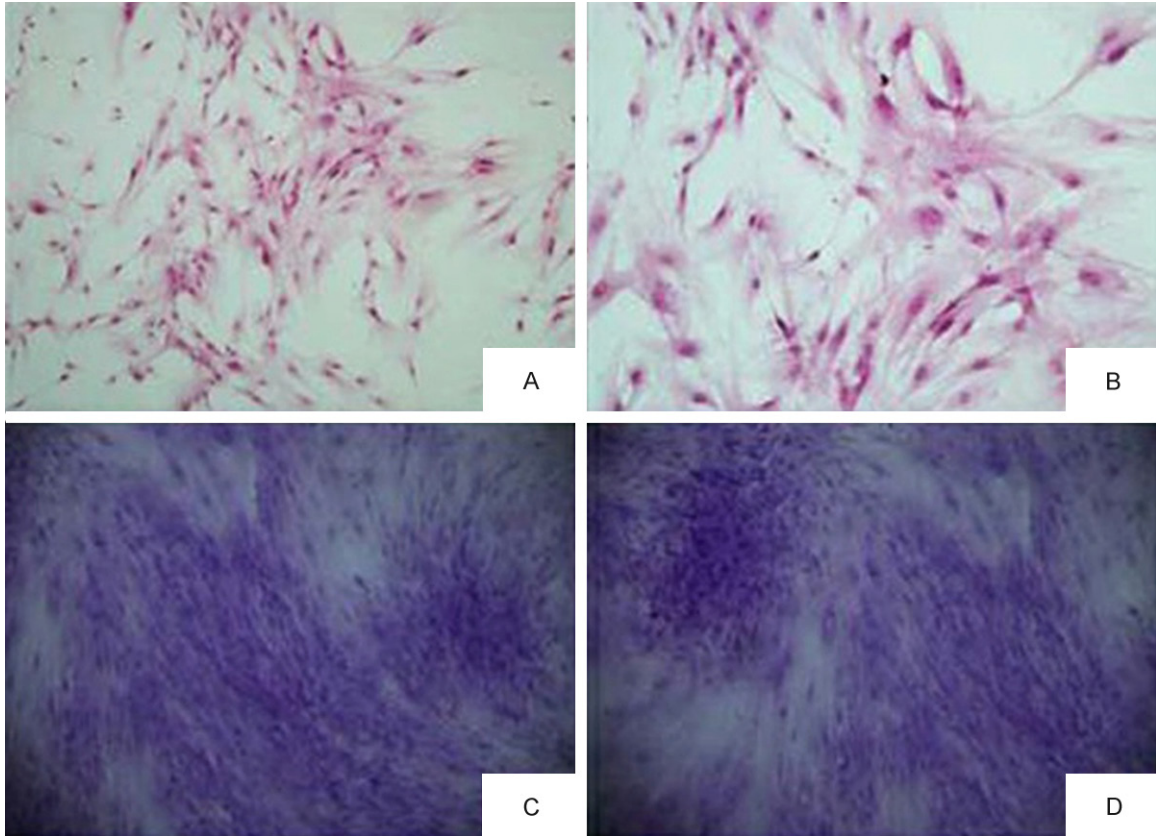


Figure 3. Hematoxylin-eosin staining results of human primary lung cancer cells *in vitro*. A, B: Hematoxylin-eosin (H-E) staining for adenocarcinoma cancer cells (100× and 200×); C, D: H-E staining for squamous cancer cells (100× and 200×).

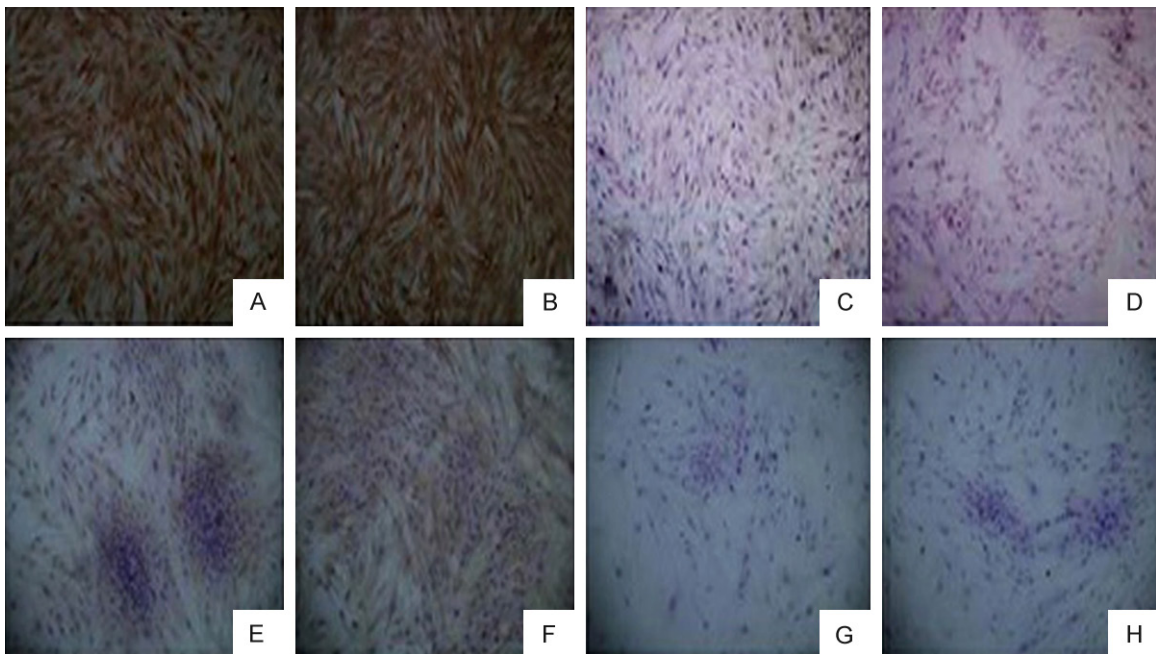


Figure 4. Immunocytochemistry analysis for human primary lung cancer cells *in vitro*. The immunocytochemistry results of human cultured primary lung cancer cells *in vitro*. A, E: CK7 staining; B, F: CK19 staining; C, G: Vimentin staining; D, H: Isotype control. A-D: Adenocarcinoma; E-H: Squamous carcinoma (100×).

Human primary lung cancer cell culture

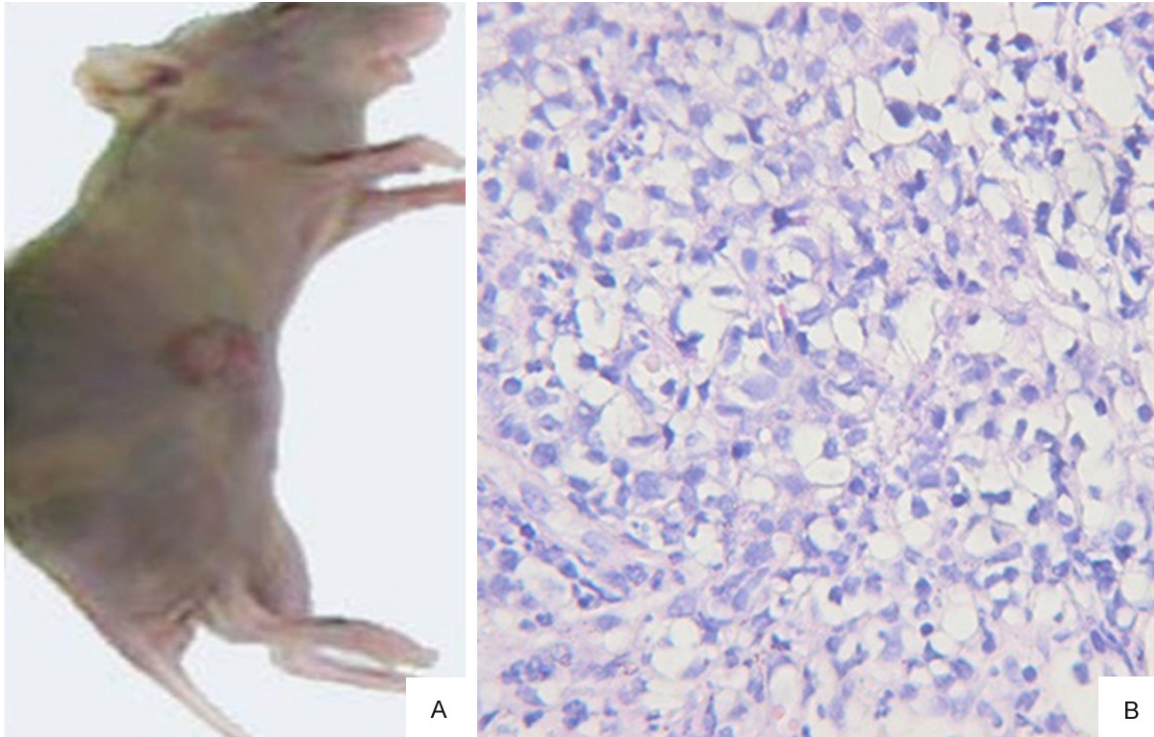


Figure 5. The results of Experiment of nude mice inoculation Experiment. A: The representative picture of the transplanted xenografts; B: H&E staining of the tumors from xenografts.

and accounted for 70-80% area of the bottom. The cell passage was usually performed after 3-4 day culture and the contaminated cells were gradually eliminated with the repeated passage. There was a positive relationship of cell morphology to cell density. The cells were in the shape of polygon when in a lower density, while they got lengthened and became fusiform when in a higher density. The cells had a strong proliferative ability even after continuous culture *in vitro* for three months and more than ten times passage (**Figure 1**).

Morphology of primary cultured human lung cancer cells in vitro

Under optical inverted microscope, the cells, presenting polygon or fusiformis, gathered together and the contact inhibition completely disappeared. The result of hematoxylin-eosin (HE) staining showed that the cells exhibited pathological mitotic figure, including a large and deeply dyed nucleus, multi-nucleoli, nuclear division and the increase of the ratio of nucleus to cytoplasm (**Figures 2 and 3**).

Immunocytochemistry (ICC) identification

The results of immunocytochemistry (ICC) revealed that after ten times of passage, the *in vitro* cultured cells were highly purified. The cells were negative for vimentin while positive for cytokeratin (CK) 7 and 19, the biomarkers for epithelial-derived tissue and non-small-cell lung cancer (NSCLC) [9] (**Figure 4**).

Experiment of nude mice inoculation

The tumor formation ability of these primary cultured cells was checked by nude mice inoculation experiment. It was demonstrated that the cancer cells readily formed subcutaneous tumors (0.3 cm×0.5 cm×0.2 cm) in nude mice 3 weeks following inoculation. The results of HE staining demonstrated that the pathological images of the transplanted tumor were consistent with its tumor origin (**Figure 5**).

Discussion

Isolation and culture of solid tumor cells under *in vitro* environment similar to the microenvi-

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ronment of the original tumor is always a challenge and requires specialized techniques [5, 10, 11]. There are also several methods for cell primary culture and each method has its own advantages and disadvantages [5]. Lung cancer tissues are rich in stromal elements and the removal of fibroblast pollution is the key to successful lung cancer cell cultivation, therefore, in this study we used collagenase to isolate the primary lung cancer cells. It was shown in present study that an ideal cell number could be obtained with the usage of incubation with 1% type IV collagenase for 1 hour and this incubation duration did not bring damage to adhesive or proliferative ability of the isolated cells. With this method, we successfully isolated and cultured the carcinoma cells from five lung cancer patients and these cells could grow and aggregate in a short time.

The selection of culture medium for *in vitro* culture of lung cancer cells is another essential factor. For example, HITES and ACL-4 can be used for SCLC and adenocarcinoma culture, respectively [12]. RPMI-1640 is one of the most common culture mediums and widely used in various cell cultivation, especially for cancer cells with a proliferation rate [12]. Thus, in this study, we used RPMI-1640 containing penicillin and streptomycin as the culture medium. It was demonstrated that a relatively high survival rate could be achieved in different types of lung cancer cells (adenocarcinoma, squamous carcinoma and adenosquamous carcinoma). The identification results showed that the *in vitro* cultured cells displayed typical morphology characteristics of malignant cells and they could continuously proliferate and be passaged for a long time when cultured *in vitro*. Moreover, the animal experiment clearly showed that the inoculated cancer cells could form transplantable tumor in nude mice and the histopathological features of the transplanted tumor were consistent with the primary tumor.

According to our experience, the following several steps are beneficial to the successful cultivation of primary lung cancer cells: (1) Avoidance of pollution: an adequate pre-preparation for tissue collection and cell culture, including sterilization of experimental equipments, an ultra clean cabinet superclean bench and aseptic technique, is prerequisite. (2) Tissue collection: the collected tissues should be rich in parenchymal tumor cells far away

from the normal tissues and without necrosis or fibrosis lesions. The failure of the culture for SCLC was attributed to the limitation of cancer cell numbers. Thus, it is important to obtain enough cancer tissues. (3) Cell culture performance: the cell isolation procedure should be performed as soon as possible after the tissue collection and the cells should be seeded on the culture bottle within 4-6 hours. (4) Removal of contaminating cells: it is important that the fibroblast cells should be eliminated to reduce their negative influence on the growth of tumor cells. In this study, to achieve the purification of cancer cells, we used trypsin digestion combined with differential adhesion method to get rid of the fibroblasts.

The significance of lung cancer cell primary culture lies in: first, different from the cell lines who have underwent genetic aberrations with increasing passage numbers, the biological characteristics of primary cultured cells are very close to their *in vivo* conditions, which can provide an ideal model for many experiments. Second, the research on the biological behavior of primary lung cancer cells is beneficial to understanding the occurrence, development and prognosis of this disease [13]. The last but not the least, via drug sensitive test and animal inoculation experiments of the primary cultured lung cancer cells; we can obtain invaluable information on the personalized treatment for cancer patients.

In summary, we have achieved the successful primary culture of human lung cancer cells via collagenase digestion. The isolated cells showed a high survival and proliferative ability in a continuous culture *in vitro*, which provides an ideal model for lung cancer research.

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Disclosure of conflict of interest

None.

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