Orthotopic Lung Cancer Murine Model by Nonoperative Transbronchial Approach

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Purpose. The aim of this work was to establish a novel orthotopic human non-small cell lung cancer (NSCLC) murine xenograft model by a nonsurgical, transbronchial approach.

Description. Male athymic nude mice and human NSCLC cell lines, including A549, H460, and H520 were used. Under direct visualization of the vocal cords, a 23-gauge blunt-tipped slightly curved metal catheter was introduced into the trachea to the bronchus, and $2.5 \times 10^5$ tumor cells mixed with Matrigel (BD Biosciences, Mississauga, Ontario, Canada) were administered into the lung. Mice were monitored using weekly microcomputed tomography scans for tumor formation.

Evaluation. When the tumor size reached more than 4 mm in diameter, the animals were euthanized, and the tumor tissue was evaluated histopathologically. Of 37 mice studied, 34 were confirmed to have tumor formation: 29 developed solitary tumors and 5 had multifocal lesions. There was no evidence of extrapleural dissemination or effusion.

Conclusions. Transbronchial delivery of tumor cells enabled the establishment of a novel orthotopic human NSCLC murine xenograft model. This clinically relevant preclinical model bearing a solitary nodule is of value for a variety of in vivo research studies.


Establishment of a clinically relevant animal model harboring human cancer is crucial for basic and translational research [1]. Currently available orthotopic human non-small cell lung cancer (NSCLC) xenograft models use percutaneous or transthoracic delivery of tumor cells, which may result in pneumothorax and pleural dissemination [2–4]. Transtracheal injection, with or without tracheostomy, may also carry a risk of causing development of multifocal lesions [5, 6]. Transbronchial instillation has the advantage of enabling development of solitary orthotopic tumors in mice, but currently reported techniques require invasive tracheostomies [7]. A clinically relevant in vivo platform to achieve solitary tumors without a surgical scar would be ideal for studies of imaging, tumor biology, and novel therapeutic interventions in lung cancer. The aim of the present work was to establish a nonsurgical orthotopic human NSCLC xenograft model in immunodeficient mice by a minimally invasive transbronchial approach.

Cell Culture and Preparation for Instillation
Human NSCLC cell lines from primary culture, including A549 (adenocarcinoma), NCI-H460 (large cell carcinoma), and NCI-H520 (squamous cell carcinoma), were provided by Dr Ming Tsao of the Department of Pathology, Princess Margaret Hospital, Toronto. Green fluorescent protein (GFP)-expressing tumor cells (H460-GFP) transfected in-house were also used. Cells were cultured in humidified incubators at 37°C and 5% CO2. Dulbecco Modified Eagle Medium (Life Technologies Inc, Burlington, Ontario, Canada) was used for the A549 cells and Roswell Park Memorial Institute 1640 medium (Life Technologies Inc) was used for the H460 and H520 cells. The media were also supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin (Pen Strep; Life Technologies Inc). Cell growth was observed daily using an inverted microscope, and cells were harvested at 70% confluence in 100-mm cell culture dishes (BD Biosciences, Mississauga, ON, Canada). Tumor cells were retrieved by 10-minute exposure to 0.25% trypsin-ethylenediaminetetraacetic acid (Life Technologies Inc), terminated by culture medium containing 10% FBS.
The viable tumor cell number was counted by trypan blue staining, and the concentration was adjusted to 1.0 × 10^5 cells/mL. Finally, the tumor cells were mixed with 1 mL growth factor-reduced Matrigel (BD Biosciences), and 50 μL tumor suspension containing 5.0 × 10^5 cells was used for instillation.

**Technique**

All animal studies were done under Animal Care Committee protocol (AUP 2150) from the Institutional Animal Care and Use Committee of the University Health Network, Toronto, ON, Canada. Male athymic nude mice (NCr-Foxn1nu; age 4 to 6 weeks; Taconic Farms Inc, Hudson, NY) were anesthetized by an intramuscular injection of ketamine (60 mg/kg) and xylazine (7 mg/kg) and maintained by 2% isoflurane inhalation. The animal was positioned supine recumbent with the head elevated 90 degrees using a rubber band attached to the front upper teeth. Pulling the tongue laterally using a Mosquito-pean forceps raised the lower jaw and exposed the glottis. The glottis and vocal cords were easily visualized using a surgical microscope with 20 magnification vision in a fixed position.

Under direct visualization of the vocal cords using a surgical microscope, a 23-gauge 2.5-cm blunt-tip slightly curved metal catheter (Harvard Apparatus, Holliston, MA) was introduced into the trachea (Fig 1A). We advanced the entire length of the catheter, and it reached the lower lobe. We intentionally tried to create the tumor in the right lower lobe in this study. A total of 50 μL tumor cell suspension (5.0 × 10^5 tumor cells) was mixed with Matrigel (Figs 1B, 1C) and administered into the lung (Fig 1D). Total procedure time, including anesthesia, was 15 to 20 min. After instillation, the mouse was given 100% oxygen, and the head was kept elevated at a 45-degree angle until recovery and return to the vivarium.

**Follow-Up Tumor Formation**

Tumor formation was monitored using weekly microCT scans (Fig. 2A) with a GE Locus Ultra scanner (GE Healthcare, Milwaukee, WI). High-frequency (40-MHz) ultrasound imaging (MS-550D; VisualSonics, Toronto, Ontario, Canada) was also used (Fig 2B) in some mice. When the tumor size reached more than 4 mm in diameter in the long axis, the animals were euthanized using carbon dioxide, and the lungs were resected and evaluated histopathologically. GFP fluorescence of H460-GFP cells was observed using an IVIS Spectrum whole-body imager (PerkinElmer, Waltham, MA) with the following settings: integration time, 5 seconds; binning factor, 4 ×; excitation filter, 465/30 nm (450 to 480 nm); and emission filter, 520/20 nm (510 to 530 nm; Fig 4).

**Pathologic Evaluation**

Immediately after resection, the lungs were fixed in 10% neutral buffered formalin and embedded in paraffin. Blocks were cut at a thickness of 4 μm, and sections were placed on silane-coated slides. The samples were stained with hematoxylin and eosin and elastic trichrome. Immunohistochemistry to evaluate tumor vasculature and epidermal growth factor receptor (EGFR) expression was performed using an anti-cluster of differentiation (CD)31 antibody (platelet endothelial cell adhesion molecule-1 antibody [M-20], 1:2500 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA) and an anti-EGFR antibody (Mouse anti-EGFr, 1:100 dilution; Life Technologies Inc), respectively, using standard protocols.

**Clinical Experience**

Of 37 mice studied (A549, n = 28; H460, n = 3; H460-GFP, n = 3; H520, n = 3), 34 were confirmed to have tumor formation. Three mice died in early stages related to the anesthesia and inoculation procedures. Twenty-nine developed solitary tumors in the developed in the right lower lobe, and multifocal lesions occurred in 5 mice (3 of 28 A549 mice [10.7%] and 2 of 6 H460 mice [33.3%]). The tumor growth curves are shown in Figure 3.

Gross inspection of the thoracic cavity showed no evidence of extrapleural dissemination or effusion in any animals (Fig 4A). The absence of extrapleural dissemination was also confirmed by GFP observation for H460-GFP tumors (Fig 4B). Because there was no surgical scar
on the skin surface, that tumor formation could easily be observed by high-frequency ultrasound imaging (Fig 2B).

In hematoxylin and eosin and elastic trichrome-stained sections, A549 tumor cells were observed growing primarily within alveolar spaces (Figs 5A, 5B, 5C). H460 tumors likewise showed tumor cells growing within airspaces, but also exhibited some invasion into bronchi and visceral pleura (Fig 5D, E). H520 tumors also showed some areas of invasion outward from bronchial lumens (Fig 5F). Differential EGFR expression was observed between each cell type by immunohistochemistry, with moderate EGFR expression for A549, weaker expression for H460, and no expression for H520 (Fig 6), consistent with the known expression profiles of these tumors.

Comment

A novel, minimally invasive, nonsurgical technique of intubation and transbronchial instillation of human NSCLC tumor cells enabled the establishment of...
orthotopic human lung cancer xenografts in immunodeficient mice with a high engraftment rate of 91.9%. The tumors show locoregional growth and are predominantly solitary, which are distinct advantages for interventional studies. A further advantage is that tumors can be grown in a specific lobar area of the lung (right or left lower lobe), without causing pleural dissemination or effusion. Further, this model will not be affected by artifacts that may be caused by other invasive implantation procedures such as percutaneous injection and thoracotomy. Complications of such invasive procedures, such as pneumothorax and bleeding, are also avoided. Finally, the mice do not incur surgical wounds, thus minimizing artifacts in imaging studies caused by overlying tissue damage or inflammation.

Endotracheal intubation techniques for mice have previously been described [8, 9]. The surgical microscope provides a very clear view of the vocal cords and easy manipulation under direct vision. By use of a 23-gauge, 2.5-cm curved blunt-tip catheter for inoculation, we can make a solitary tumor in the right or left lower lobe. We can observe tumor formation in the right or left lower lobe and obtain tissue samples for arbitrary steps of tumor genesis. Furthermore, this model can be used to observe lung cancer growth in the true orthotopic lung tissue microenvironment, which is increasingly recognized to play a major role in promoting or inhibiting tumor growth [2]. By using different subtypes of tumor cells, the biologic behavior of specific tumor cells can be observed under clinically relevant conditions, and the influence of specific gene expression can be evaluated. Actually, the 3 cell lines expressed different levels of EGFR protein, which is one of the important targets in the field of molecularly targeted therapeutic agents [10].

Fig 5. Histologic features of the different tumor types: A549 tumor with (A) hematoxylin and eosin (H&E stain; original magnification ×25), (B) elastic trichrome stain (original magnification ×200), and (C) cluster of differentiation (CD)31 stain (original magnification ×200); H460 tumor with (D) H&E stain (original magnification ×100) and (E) elastic trichrome stain (original magnification ×100); and (F) H520 tumor with H&E stain (original magnification ×200).

Fig 6. Immunohistochemistry for epidermal growth factor receptor showing different expression patterns between the tumor types, with (A) strong positive expression for A549 (original magnification ×200), (B) weaker expression for H460 (original magnification ×200), and (C) minimal expression for H520 (original magnification ×400).
This model provides a powerful in vivo platform for a range of investigations, including imaging, tumor biology, and monitoring of responses to novel therapeutic interventions.

Disclosures and Freedom of Investigation

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References


Disclaimer

The Society of Thoracic Surgeons, the Southern Thoracic Surgical Association, and The Annals of Thoracic Surgery neither endorse nor discourage use of the new technology described in this article.

INVITED COMMENTARY

Doctor Nakajima and his colleagues described a challenging orthotopic human lung cancer model in athymic mice [1]. Cultured human non-small cell lung carcinoma cells were injected into a specific lung lobe with a custom-made blunt-tip thin catheter. The use of a surgical microscope for tracheal intubation ascertained the location of tumor inoculation and minimized procedure-related mortality rate. With this unique model, the authors reported different patterns of oncogene expression in tissue samples from different types of cancers.

Orthotopic models have been increasingly gaining popularity in lung cancer research aiming to replicate clinical characteristics of the diseases. One method of tumor inoculation is to inject tumor cells into the lung parenchyma or plural space. First, the model can be induced by direct injection of cells into lung parenchyma by means of thoracotomy, which provides a high tumor take rate; however, the animals experience a major surgery at the beginning of the experiment. Second, trans-thoracic injections can be performed after a smaller skin incision, which provides sufficient visualization of lung movements while keeping the plural membrane intact. Third, the model can be induced by empirical injection with a thin needle without visual guidance [2]. Another route for tumor inoculation is through the airway either by tracheotomy or by tracheal intubation [3]. In the present study, the authors advanced the merits of noninvasive tracheal intubation and aimed the location of injection to a specific lobe. The tumor take rate was nearly 100% after exclusion of the procedure-related mortality. The reproducibility of the model can be further improved by pretreatment evaluation of tumor burdens using advanced noninvasive imaging technologies, such as micro computed tomography, which was reported in the present study, as well as positron emission tomography and bioluminescent imaging providing a luciferase-transfected tumor cell line is used. One concern of the present model was whether there were feasible ways to distinguish those mice with multifocal lesions from those mice with solitary tumors at an early stage of the experiment.

In conclusion, I am very delighted to read this article and learn from this model by Nakajima and colleagues [1]. The procedure and the means of monitoring tumor progression in this model could be modified accordingly to meet the requirements of studies from basic research to translational research, including but not limited to...