Metastatic patterns of lung cancer visualized live and in process by green fluorescence protein expression

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We demonstrate here the visualization of human lung cancer metastasis live and in process in nude mice by green fluorescent protein (GFP) expression. The human lung adenocarcinoma cell line Anip 973 stably transfected with the humanized GFP-S65T cDNA was selected for very bright green fluorescence. GFP-transfected lung cancer cells were initially inoculated subcutaneously in nude mice. Five weeks after transplantation, the resulting tumor had reached over 1 cm in diameter and had very bright GFP fluorescence. Fragments of subcutaneous tumor were implanted onto the visceral pleura of the left lung of nude mice by surgical orthotopic implantation (SOI) of histologically-intact tissue via transverse thoracotomy. The ipsilateral resulting tumor was highly fluorescent due to GFP expression. GFP expression allowed the visualization of the advancing margin of the ipsilateral tumor into the fresh normal lung tissue. Lymphogenous and direct-seeding metastases in the pulmonary hilum, cervical lymph nodes, the mediastinum and contralateral pleural cavity and contralateral lung in the SOI-treated mice were brightly visualized by GFP expression in fresh tissue. GFP-transfected and untransfected tumor had similar metastatic characteristics suggesting that GFP expression had no effect on metastasis itself. The results with the GFP-transfected tumor cells, combined with the use of SOI, demonstrate a fundamental advance in the visualization and study of lung cancer metastasis in process.

Keywords: lung cancer, green fluorescent protein, metastatic process, nude mice, surgical orthotopic implantation

Introduction

Approximately 150,000 new cases of lung cancer will be diagnosed each year in the USA alone, where it is the number one cancer killer [1]. In Japan, lung cancer has surpassed stomach cancer as the most frequently occurring cancer. Since 1950, the death rate from lung cancer has increased over 200% among males and over 300% among females [1].

Tobacco smoking accounts for 90% of lung cancer. The predominant cell type of lung cancer is adenocarcinoma. In adenocarcinoma, there is preferential spread to the opposite lung [1]. During this usual lethal process, the tumor can extend to the hilar region, the mediastinum, the contralateral pleural cavity, as well as local and regional lymph nodes with possible widely disseminated disease resulting.

The process of metastasis consists of a series of interactions between tumor cells and host cells or tissues [2]. However, the process has been difficult to analyse because of lack of suitable experi-
mental models and lack of high resolution histological analytical tools that can be used on living tissue. Previous studies of metastasis have been hampered by the inability to identify small numbers of tumor cells in a background of host cells in the living state. Previous techniques were not sufficiently informative as to how tumor cells spread from a primary tumor leaving us with a relatively poor understanding of metastasis [2]. To understand how tumor cells metastasize and progress in the target organs, what has been needed is an appropriate model and a way to directly visualize the process in vivo. We have developed orthotopic transplantation models that use surgical orthotopic implantation (SOI) of histologically intact tissue [3–5]. These models have been developed for all tumor types including lung which involves the use of transverse thoracotomy on nude mice to transplant human lung tumor tissue directly on the mouse lung [3–5]. These models remarkably replicate the clinical behavior of human cancer including metastasis.

To enhance the resolution of the visualization of metastasis in process, we have utilized the green fluorescent protein (GFP) gene. GFP cDNA encodes a 283 amino acid polypeptide with a molecular weight of 27 kDa [6]. The humanized clone hGFP-S65T was isolated to develop brighter and higher gene expression in human cells [7–9]. We describe in this report the use of stable transfectants of human lung adenocarcinoma cells (Anip 973) that express high-level GFP fluorescence in vivo to study human lung cancer metastasis to the contralateral lung in the SOI nude-mouse model [3–5, 10].

Materials and methods

Cell line and animals
Human lung cancer cell (Anip 973) was obtained from Harbin Medical University, Harbin, China. Six-week-old BALB/c nu/nu male mice were used for in vivo animal experiments.

DNA manipulations and expression vector constructions
The dicistronic expression vector (pED-mtx') was obtained from Genetics Institute (Cambridge, MA). The expression vector containing the codon-optimized hGFP-S65T gene was purchased from CLONTECH Laboratories Inc. (Palo Alto, CA). To construct the hGFP-S65T-containing expression vector, phGFP-S65T was digested with Hind III blunted at the end. The entire hGFP coding region was then excised with Xba I. The pED-mtx' vector was digested with Pst I, blunted at the end, and further digested with Xba I. The hGFP-S65T cDNA fragment was then unidirectionally subcloned into pED-mtx'.

Cell culture, transfection, subcloning
Anip 973 cells were cultured in RPMI-1640 (Gibco) containing 10% fetal calf serum (FCS; Gemini Bio-products, Calabasas, CA), 2 mM L-glutamine and 100 μM non-essential amino acids (Irwin Scientific, Santa Ana, CA). For transfection, near-confluent Anip 973 cells were incubated with a precipitated mixture of LipofectAMINE™ Reagent (Gibco), and saturating amounts of plasmids for 6 h before being replenished with fresh medium. Anip 973 cells were harvested by trypsin/EDTA 48 h post-transfection and subcultured at a ratio of 1:15 into selective medium which contained 50 μM methotrexate (MTX). Cells with stably integrated plasmids were selected by growing transiently-transfected cells in the MTX-containing medium. Clones were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA. The clones were amplified and transferred with conventional culture methods. Clone-26 was chosen because of its high-intensity GFP fluorescence and stability.

Subcutaneous human lung tumor growth in nude mice
Nude mice were inoculated subcutaneously with a single dose of 10⁷ Clone-26 cells. Cells were first harvested by trypsinization and washed three times with cold serum-containing medium, then kept on ice. Cells were inoculated in a total volume of 0.4 ml within 40 min of harvesting. Mice were sacrificed to obtain tumor fragments when the tumor had reached approximately 1.2 cm in diameter.

Surgical orthotopic implantation of human lung cancer in nude mice
Tumor pieces (1-mm³) derived from the nude mouse subcutaneous tumor formed from Clone-26 were subsequently implanted by surgical orthotopic implantation (SOI) onto the left visceral pleura in four nude mice [3–5, 10]. The mice were anesthetized by Isofluran inhalation. A small 1-cm transverse incision was made on the left-lateral chest of the nude mice via the fourth intercostal space. The small incision provided access to the pleural space, and resulted in total lung collapse. Five tumor pieces were sewn together with a 8-0 nylon (Look, Norwell, MA) surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung with one suture. The lung tissue was then returned into the
GFP visualized metastatic process in fresh tissue

Figure 1. The human lung adenocarcinoma cell line Anip 973 transfected with the pED-mtx' vector in which the hGFP-S65T and DHFR genes were transcribed in a dicistronic message is shown in vitro. The stable high-expression Clone-26 was selected in 50 nM MTX. (Confocal microscopy, original magnification ×100.)

image was captured with a Pixera camera (1260 x 960 pixels) and was processed with IRAF (Image Reduction and Analysis Facility) software (National Optical Astronomy Observatory, Tucson, AZ).

Results

Transfection of GFP expression vector to human lung cancer cells

The GFP expression-vector transfected cells were able to grow in levels of MTX up to 50 nM. The subcloned MTX-resistant Anip 973 cells showed a striking increase in GFP fluorescence compared to the transiently-transfected cells. Figure 1 is a confocal micrograph of a subclone which expressed the strongest GFP which was isolated and termed Anip 973-hGFP-S65T-Clone-26 (Clone 26). Note that essentially 100% of the tumor cells are expressing GFP as seen in this digital image captured by confocal microscopy.

GFP expression in subcutaneous tumors in nude mice

A > 1 cm tumor was formed 5 weeks after inoculation of 1 × 10^7 Clone-26 cells on the flank of a nude mouse and was used as stock tissue for surgical orthotopic implantation to the nude mouse lung. This tumor fluoresced very brightly (data not shown).

Patterns of lung tumor metastases after SOI visualized by GFP expression

Primary tumor grew in the operated left lung in all mice after SOI (Figure 2A, B). Figure 2A shows the fluorescence of the operated left lung. As seen in Figure 2B, GFP expression allows visualization of the advancing margin of the tumor spreading in the ipsilateral lung. All four animals explored had evidence of chest wall invasion and local and regional spread. Metastatic contralateral tumors involved the mediastinum, contralateral pleural cavity (Figure 2C, D), and the contralateral visceral pleura (Figure 2E), occurring in all four mice. Figure 2A and B compare the ipsilateral lung tumor on the one hand with the contralateral lung metastasis on the other hand (Figure 2E). While the ipsilateral tumor has a continuous and advancing margin, the contralateral tumor seems to have been formed by multiple seeding events. These observations were made possible by GFP fluorescence of the fresh tumor tissue.

When non-GFP-transfected Anip was compared with GFP-transformed Anip for metastatic capability similar results were seen. For example, Figure 2F shows that non-GFP-transfected Anip can metastasize to the contralateral lung after SOI.
Cervical lymph nodes were also involved as well as contralateral hilar lymph nodes shown by GFP expression. Hilar lymph node metastases were found in four of four mice explored. A lone cervical lymph node metastasis was found in one of four mice explored, brightly visualized by GFP fluorescence (Figure 3A, 3B). However, there was no tumor involvement in the brain, liver, spleen, kidneys, adrenal glands, or para-aortic lymph nodes.

Figure 2. (A) Spreading tumor in the mediastinum. Bar = 3 mm. (B) Spreading tumor in the ipsilateral lung with margin highly visualized by GFP expression. A small vessel developed at the margin of the tumor (white arrowheads). Bar = 500 μm. (C) Primary tumor involved the mediastinum directly. Tumor also involved the contralateral pleural cavity. Arrowheads indicate the esophagus in the mediastinum. (D) Tumor grew with high GFP expression as can be visualized by bright fluorescence in the same field as (C). Bar = 3 mm. (E) Spreading tumors which ranged from 1.2 to 3.7 mm in diameter in the contralateral visceral pleura. Arrowheads indicate the fluorescent tumors. Bar = 3 mm. (F) Tumor growth in the contralateral right lung after SOI of non-GFP-transformed Anip tissue in the left lung. Thin arrows indicate contralateral tumor. Thick arrow indicates contralateral right lung. Bar = 3 mm.
Discussion

We have demonstrated the metastatic pattern of human lung adenocarcinoma visualized in exquisite detail by GFP expression in fresh tissue which eliminates all the artifacts of fixation or freezing.

Previous studies have used external fluorescent tags [10, 11] or reporter genes such as lac-Z [13] to follow metastases. However, the former will not be passed on to many progeny cells and the latter requires staining. To overcome these problems, we visualized cancer metastasis in fresh tissue and over time by GFP expression. Figure 3 shows the possibility of high resolution visualization of tumor metastasis. Even metastases which are <100 μm in diameter can be visualized easily and clearly by fluorescence microscopy. Such small colonies would be very difficult to visualize in bright field microscopy in fresh live tissue or even in stained paraffin sections.

GFP-gene transfecants can be followed in the primary and target organs, since the fluorescent gene has been integrated and is passed on to the daughter cells. The GFP-expressing cells can be visualized in fresh live tissues without any distorting histological preparation and interference from endogenous GFP. For the first time, we can now visualize tumor cells growing and spreading in vivo in their natural state.

The fact that GFP and non-GFP-transformed Anip have similar metastatic potential such as metastasis to the contralateral lung (Figure 2F), indicates that GFP expression does not influence the basic metastatic potential of the human tumor cells.

Standard fluorescent microscopy allows high-resolution visualization of the GFP-expressing tumor. The SOI model allows full expression of the tumor's metastatic potential [3-5, 10] and represents the entire process of the metastasis, including local tumor growth, as well as vascular and lymphatic invasion at the local site. In this case, we utilized the SOI model and GFP-expressing tumor to visualize human lung tumor growth and metastases to the mediastinum and contralateral lung, as well as to lymph nodes. This is a very important clinical event and can now be visualized much more realistically.

GFP fluorescence will facilitate the understanding of metastatic processes including each step of the metastatic cascade. These results provide us with new insights into the metastatic process of lung cancer such as the striking difference of the patterns of tumor growth in the ipsilateral (Figure 2A, B) and contralateral lungs (Figure 2E) which should contribute to our understanding of metastasis and development of new therapy. The results with GFP-transfected tumor cells demonstrate a fundamental new technology to effectively study, for the first time, cancer metastasis in the fresh living state.

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References