Comparison of GFP-Expressing Imageable Mouse Models of Human Esophageal Squamous Cell Carcinoma Established in Various Anatomical Sites

TAO HU1,3, HUI QI2, PEI LI1, GUOQIANG ZHAO1, YANGCHENG MA1, QIANYUN HAO1, CHUNZHI GAO1, YILIN ZHANG1, CHUNYAO WANG3, MENG YANG2,5, ROBERT M. HOFFMAN4,5*, PING CHEN1* and ZIMING DONG1

1College of Basic Medical Sciences, Zhengzhou University; Collaborative Innovation Center of Henan province for cancer chemoprevention, Zhengzhou, P.R. China; 2AntiCancer Biotech (Beijing) Co., Ltd., Beijing, P.R. China; 3Laboratory Animal Center, Zhengzhou University, Zhengzhou, P.R. China; 4Department of Surgery, University of California, San Diego, CA, U.S.A.; and 5AntiCancer, Inc., San Diego, CA, U.S.A.

Abstract. Background/Aims: Esophageal squamous cell carcinoma (ESCC) is a recalcitrant cancer. Mouse models of this disease could be used for discovery of more effective therapy for ESCC. Materials and Methods: The green fluorescent protein (GFP)-expressing human esophageal cancer EC1 cell line was established with a lentiviral expression system. Subsequently, nude mice were injected subcutaneously, intracardiac or intravenously, or orthotopically implanted with EC1-GFP cells. Tumor growth and metastasis were examined by fluorescence imaging in vivo or by open fluorescence imaging after autopsy. Results: Four different mouse xenograft models of ESCC expressing GFP were established. In the subcutaneous model, primary tumor growth was monitored in real-time by whole-body fluorescence imaging. No metastasis was observed in the subcutaneous or surgical orthotopic implantation model. By 55 days after implantation, all mice had developed orthotopic esophageal cancer, but without detectable metastasis. In contrast, experimental metastasis occurred in the intracardiac and intravenous models. In the intravenous injection model, the lung was the sole organ of experimental metastasis. In the intracardiac model, extensive experimental metastases occurred in the bone, brain and lung. Conclusion: The mouse xenograft models of ESCC developed in the present study can provide a means of discovering more effective therapy of this recalcitrant type of cancer.

Esophageal carcinoma is a recalcitrant cancer with surgical resection as the only effective current treatment. However, long-term survival rarely occurs when lymph-node or distant-organ metastases have occurred (1).

We previously developed an aggressive orthotopic model of the human esophageal adenocarcinoma cell line PT1590, stably transfected with green fluorescent protein (GFP). Orthotopic tumor growth, as well as metastatic spread, was visualized by fluorescence imaging and high-resolution MRI. Highly aggressive fluorescent cell lines were isolated from metastatic tissues, cultured and re-implanted orthotopically. Metastasis to liver, lung and lymph-nodes occurred in up to 83% of the animals (1).

We subsequently evaluated the response to trastuzumab therapy in the OE19 orthotopic human esophageal adenocarcinoma nude mouse model, overexpressing human epidermal growth factor receptor 2 (HER-2). Anti-HER-2 therapy with trastuzumab resulted in tumor growth reduction and a decrease of lymph-node metastases in this orthotopic model (2).

We also developed an orthotopic nude mouse model of peritoneal carcinomatosis of PT1590-GFP esophageal carcinoma. In this model, 50% of the animals developed...
extensive peritoneal dissemination. Metastasis to the liver, lungs, and lymph-nodes also occurred (3).

The efficacy of inhibition of HER-2 and CXCR4 receptor pathways was also determined in the orthotopic model of esophageal carcinoma. There was a correlation between HER-2 and CXCR4 expression in primary tumors and metastases in the orthotopic model. Trastuzumab and AMD3100 treatment led to a significant reduction of primary tumor growth, metastases and micrometastases (4).

Esophageal squamous cell carcinoma (ESCC) is a recalcitrant cancer. Mouse models of this disease could be used for discovery of more effective therapy for ESCC. The GFP-expressing human esophageal cancer EC1 cell line was established with a lentiviral expression system. Subsequently, nude mice were injected subcutaneously, intracardiac or intravenously, or orthotopically implanted with EC1-GFP cells. Tumor growth and metastasis were examined by fluorescence in vivo imaging or by open fluorescence imaging after autopsy in each model.

Materials and Methods

Mice and reagents. Mouse experiments were approved by the Institutional Animal Care and Use Committee of Zhengzhou University (approval number ACB20140007). Balb/c nude female mice were purchased from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China). The mice were housed in individual ventilated cages under sterile conditions. Sterile food and water were provided ad libitum. Mice aged 5 to 6 weeks were used for the subcutaneous, intracardiac or intravenous injection or orthotopic implantation of cancer cells. For surgical manipulation, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight).

Generation of a stable GFP-expressing EC1 cell line. EC1 cells used in the present study were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) at 37°C and 5% CO2. For GFP gene transduction, 20% confluent EC1 cells were incubated with GFP-lentivirus supernatants from the 293T packaging cells for 72 h. High GFP-expression cell clones were selected by limited-dilution assays. Cell clones were visualized by fluorescence microscopy to detect GFP. The brightest 5 to 10% of the population was further sorted by fluorescence activated cell sorting. The sorted cells were enriched, cultured, and further analyzed by flow cytometry (5).

Cell growth curves. EC1-GFP and parental EC1 cells in exponential growth phase were trypsinized to a single-cell suspension. Cells were seeded in 96-well plates (3x10³ cells per well). Cell number was determined with the MTT Assay (Sigma, St. Louis, MO) according to the manufacturer’s instructions.

Figure 1. Establishment and characterization of a stable EC1 cell line with high level expression of green fluorescent protein (GFP) (EC1-GFP). A: Fluorescence and bright-field images of the EC1-GFP cells. B: Fluorescence-activated cell sorting analysis of the percentage of fluorescent cells. C: A comparison of the growth curves of parental EC1 and EC1-GFP cells. D: Cell invasion assays were used to compare EC1 and EC1-GFP cells. Average optical density (OD) values (±standard deviation) detecting invasion from three independent experiments. Images of invading cells and bar graphs of their numbers are shown. Original magnification, ×200.
In vitro invasion assay. The Matrigel™ invasion assay was performed according to previous reports (6). Transwell® cell culture inserts (Corning Inc., Shanghai, China), with a filter-membrane pore size of 8 μm, were used in the assay, in the absence or presence of 50 μl matrigel (BD). EC1-GFP and parental EC1 cells in serum-free RPMI-1640 medium (2×10⁵ cells/100 μl) were added to the upper compartment of the chamber, and 800 μl of conditioned medium was added to the lower compartment. After 24-h incubation, cells from the upper surface of the filter were removed. Invasive cells that had passed through to the lower surface of the filter were fixed and stained with 0.2% crystal violet solution (Sigma). The chamber was then washed with phosphate buffer saline (PBS) and photographed at ×200 magnification. Crystal violet was placed on the transwell membrane, dissolved in 33% acetic acid solution, and absorbance was measured at OD570 nm.

Establishment of the subcutaneous xenograft model. EC1-GFP cells were harvested using 0.25% trypsin-EDTA and a single-cell suspension (5×10⁶/100 μl) was injected subcutaneously into the left flank of 10 Balb/c nude female mice. Tumor growth was observed and tumor area was recorded twice a week with a FluorVivo Model-300 imaging system (Indec Systems, Santa Clara, CA) (7, 8). At the same time, tumor size was measured with calipers and tumor volume was calculated according to the formula: (shortest diameter)² × longest diameter ×0.5. When the tumors reached approximately 1000 mm³ (36 days after inoculation), the mice were euthanized by CO₂ inhalation. Then all mice were dissected and open fluorescence imaging was performed. The organs and lymph-nodes were dissected and removed for further examination for metastasis using fluorescence imaging. The subcutaneous tumors were excised and half of the tissue specimens were formalin-fixed and paraffin-embedded for hematoxylin and eosin staining.

Surgical orthotopic implantation (SOI) model. The SOI model of ESCC was performed according to previous reports with some modifications (9, 10). Exponentially growing EC1-GFP cells (1×10⁷

Figure 2. Monitoring tumor progression after subcutaneous implantation of EC1-GFP cells. A: Real-time in vivo imaging of growth of EC1-GFP tumors. Panels represent sequential fluorescence imaging of a single mouse taken on the days indicated after subcutaneous injection of EC1-GFP cells. B: The subcutaneous tumor was observed using whole-body imaging and bright-field imaging. C: The fluorescent area was recorded with the FluorVivo Model-300 imaging system twice a week and analyzed using Power Analysis Station. D: Tumor size was measured with calipers and tumor volume was calculated according to the Materials and Methods.
/100 μl) were inoculated subcutaneously into Balb/c nude female mice. At approximately 45 days post-inoculation, when the tumors had reached 1-1.5 cm in diameter, they were excised under aseptic conditions. Necrotic tissues were cut away, and the remaining tumor tissues were minced into approximately 1 mm³ pieces. For orthotopic implantation, 10 Balb/c nude female mice aged 5 to 6 weeks were anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg). A 1 cm incision was then made just at the lower edge of the left rib cage, along the edge of ribs on the left side. The intra-abdominal segment of the lower esophagus was carefully exposed, and one 1 mm³ tumor piece was transplanted onto the lower esophagus with an 8-0 monofilament nylon suture (Shanghai Pudong Jinhuan Medical Products Co., Ltd., Shanghai, China). The stomach was then returned to the peritoneal cavity and the abdominal wall and the skin was closed with 6-0 Dexon sutures (Ethicon, Inc., Somerville, NJ, USA). Animals were kept in a sterile environment. All procedures of the operation described above were performed with a 7× microscope (Olympus, Tokyo, Japan). Fifty-nine days after implantation, animals were euthanized and autopsied.

Experimental metastasis models established with intracardiac and intravenous injection of EC1-GFP cells. To establish the intracardiac injection model, a single-cell suspension of EC1-GFP
cells (2×10^6/100 μl) was injected into the left heart ventricle, according to the method described previously (11). Nineteen days after implantation, mice were sacrificed and autopsied. Another group of mice were injected in the tail vein with an equal number of EC1-GFP cells. Thirty-five days after implantation, animals were euthanized and autopsied. Tumor growth and metastasis were monitored and recorded twice a week with the FluorVivo Model-300 imaging system.

Figure 4. Metastasis of EC1-GFP cells after intracardiac injection. A: Real-time in vivo imaging of metastasis. Panels represent sequential fluorescence imaging of a single mouse taken on days 1, 4, 7, 10, 13, 16 and 19 after intracardiac injection of EC1-GFP cells. B: Representative fluorescence imaging from dorsal (left panel) and ventral (right panel) areas. C: Nineteen days after injection, all the animals were sacrificed and the skeletal system was observed with the FluorVivo imaging system (left panel) and in bright-field (right panel). D: Brain metastases were observed in bright-field (left panel) and with the FluorVivo imaging system (right panels). E: Lung metastases were observed in bright-field (left panel) and with the FluorVivo imaging system (right panels). Arrows show the metastases in the lung. F: Micrometastases in the brain were visualized under fluorescence microscopy. G: Micrometastases in the lung were visualized under fluorescence microscopy.
Imaging of tumor growth and metastasis. Whole-body images of each mouse were obtained with the FluorVivo Model-300 imaging system in live animals, twice a week as described previously (7, 8). High-resolution images were captured directly on a personal computer (Axis 945GM) and analyzed using Power Analysis Station (INDEC). At the time of sacrifice, open fluorescence imaging was performed. Primary tumor and metastatic spread were visualized and organ localization was confirmed. Organs and lymph nodes were dissected and removed and metastasis was further examined using fluorescence imaging. 

Hematoxylin and eosin (H&E) staining. Slides were cut from paraffin-embedded blocks using a microtome and stained with H&E using previously established protocols (12).

Results

Characterization of the EC1-GFP cell line. Fluorescence microscopy demonstrated that almost all cells displayed bright green fluorescence (Figure 1A). Flow cytometric analysis further showed that the percentage of GFP-positive EC1 cells was 98.7% (Figure 1B). Moreover, compared with the parental EC1 cells, it was found that GFP expression did not result in altered cell proliferation (Figure 1C) or invasive capacity (Figure 1D). These results indicated that the EC1-GFP cells were suitable for establishing animal models of ESCC.

Subcutaneous xenograft model of ESCC. One day after implantation, visible subcutaneous tumor growth of EC1-GFP was observed by fluorescence imaging in all the implanted mice (Figure 2A and B). EC1-GFP tumor size was determined by calipers and fluorescence area was measured with the FluorVivo Model-300 imaging system, twice a week (Figure 2C, D). The fluorescence area imaged in live mice correlated well with the tumor volume measured with calipers (Figure 2C, D) (13). Thirty-six days after s.c. injection of EC1-GFP cells, all the mice were sacrificed and open fluorescence imaging was performed. The organs and lymph-nodes were dissected and removed for further examination using fluorescence imaging. However, no detectable GFP metastases were found in the organs (data not shown).

Surgical orthotopic implantation model of ESCC. Six weeks after subcutaneous injection into nude mice, resulting EC1-GFP tumors (1-1.5 cm in diameter) were harvested minced and tissue fragments were transplanted onto the esophagus using SOI. All mice developed orthotopic tumors after implantation with EC1-GFP tumor fragments. Figure 3A shows the SOI procedure. When the performance status of the mice clearly decreased due to tumor progression, the mice were sacrificed and autopsied. Orthotopic tumor growth was confirmed by fluorescence and bright-field imaging (Figure 3B). The organs were dissected, removed, and further examined for metastasis using fluorescence and bright-field imaging. However, no metastasis was observed (Figure 3C).

Experimental metastasis model of ESCC with intracardiac injection. After injection of EC1-GFP cells intracardiac, bone metastases developed in 10 days (Figure 4A). Metastases to the mandible, distal femur, and proximal tibia were imaged (Figure 4A-C). 19 days after injection, all the mice were sacrificed and the main organs and skeletal system were observed with the FluorVivo imaging system. GFP fluorescence in the mandible, rib, spine, distal femur, and proximal tibia indicated metastases in these organs (Figure 4C). Metastases were also observed in the brain and lung. Representative open fluorescence images and bright-light images of the brain (Figure 4D) and of the lung (Figure 4E) are shown. Metastases were confirmed by fluorescence microscopy, in the brain (Figure 4F) and lung (Figure 4G). Metastases also occurred in the liver (1/9), mediastinal lymph-nodes (2/9), pancreas (2/9), ovary (3/9), adrenal gland (3/9) and kidneys (4/9) (Table I).
Experimental metastasis model of ESCC developed with intravenous injection. The metastatic pattern of EC1-GFP was also evaluated by tail vein injection of $2 \times 10^6$ cells into the mice. The mice were sacrificed 35 days after the injection. After sacrifice, open fluorescence imaging showed metastases, in the lung (Figure 5A). Metastases on each side of the
extracted lung were readily visualized (Figure 5B). All the mice developed lung metastases. However, no metastases to other organs were detected.

**Histopathological examination of ESCC.** After autopsy, the organs were resected and processed for routine histological examination using H&E staining. Histological analysis further characterized the subcutaneous (Figure 6A) and orthotopic (Figure 6B) tumors, and also confirmed metastases in the brain (Figure 6C), mandible (Figure 6D), distal femur, and proximal tibia (Figure 6E).

**Discussion**

Orthotopic models have been established for numerous tumor types, including esophageal cancer (1, 3, 13-17). A previous study by Gros et al. reported that 55 days after orthotopic implantation of esophageal adenocarcinoma GFP-PT1590 tumor fragments to the abdominal esophagus, there was metastatic spread to the liver in 60% of the mice and to the lung and lymph-nodes in 80% of mice (1). When Gros et al. injected GFP-PT1590 cells into the abdominal esophagus, 50% of animals developed extensive peritoneal spread without a distinct primary tumor at the injection site after 63 days (3). However, in the present study, when ESCC EC1-GFP was implanted on the abdominal esophagus, no metastases appeared, although primary tumor growth was substantial. Another model of squamous cell carcinoma of the cervical esophagus in rats achieved 99.5% tumor take with only three lymphatic micrometastases in 18 rats (16). EC1 cells, established from a human squamous cell carcinoma of the oesophagus, were highly invasive in vitro (18). Why there were no detectable metastases in the SOI model using the ESCC cell line still needs further study.

One of the leading causes of esophageal cancer death is metastasis to vital organs such as the liver, bone marrow, or lungs (19). The most common injection route employed for experimental metastasis models is the lateral tail vein of mice (20). Tail vein injection results primarily in pulmonary metastases, which is mainly because the injected cancer cells tend to be trapped in the first capillary bed in which they arrive (20). In the present study, intravenous injection of EC1-GFP cells led to multiple lung metastases without metastases in other organs.

Injection of cancer cells into the left cardiac ventricle of immunodeficient mice is widely used for experimental models of bone metastasis (11, 21, 22). In the present report, EC1-GFP cells were injected into the left cardiac ventricle. Whole-body fluorescence imaging readily detected bone metastases, especially in the mandible, distal femur, and proximal tibia, 10 days after injection. Furthermore, fluorescence imaging enabled real-time monitoring of the growth kinetics of the same animal at each metastatic site. With open imaging, metastases in the brain, lung, liver, mediastinal lymph-nodes, pancreas, ovaries, adrenal gland and kidneys were also observed (Table I). Importantly, the fluorescence signal allowed detection of single cells and microscopic metastases in the lungs, brain and other parts of the body, suggesting that this model may also be useful for monitoring the early stages of metastatic cancer colonization.

Establishing experimental models of ESCC metastasis can lead to further understanding of this disease and the discovery of more effective therapies.

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Conflicts of Interest

The Authors have no conflicts in regard to this study.

References


