Orthotopic Fluorescent Peritoneal Carcinomatosis Model of Esophageal Cancer

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Abstract. *Aim: Orthotopic models utilizing orthotopic implantation have been used for developing cancer models of multiple tumor entities. The aim of this study was to evaluate the role of orthotopic injection in establishing a model of esophageal cancer using a human green fluorescent protein (GFP) cell line of human esophageal carcinoma. Materials and Methods: Nude mice were orthotopically injected in the abdominal esophagus with stably transfected GFP-PT1590 cells. Tumor progression was examined by fluorescence imaging. Results: Fifty percent of animals developed extensive peritoneal spread without a distinct primary tumor at the injection site. Continuous and metastatic spread to the liver, lungs, and lymph nodes was also observed. Fluorescence imaging enabled fast and specific visualization of tumor progression without the need for anesthesia. Intraperitoneal and metastatic tumor spread of GFP-PT1590 esophageal carcinoma demonstrated a highly aggressive but heterogeneous behaviour. Although injection of the esophageal carcinoma cell line GFP-PT1590 did not lead to primary esophageal tumor development at the site of injection, 50% of the mice developed extensive peritoneal spread, as well as lymph node and organ metastasis. Conclusion: The orthotopic cell injection model resulted in peritoneal carcinomatosis of esophageal adenocarcinoma, which could be visualized in real time using fluorescence imaging.*

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Esophageal carcinoma is one of the leading causes of cancer-related deaths in the world (1, 2). In the advanced stages, surgical resection is not an option and treatment is limited, especially with respect to lymph node or distant organ metastases (3). To reduce primary tumor size and to effectively treat or even prevent metastatic spread, the key is to understand metastatic pathways and develop novel therapeutics. To achieve such goals, clinically relevant mouse models of the disease are necessary. Orthotopic animal tumor models, which exhibit a metastatic pattern similar to that of humans, are essential for understanding the pathophysiology of tumor disease and progression, especially in metastatic esophageal carcinoma. Animal tumor models using orthotopic injection have proven to be suitable for the evaluation of local and metastatic cancer for various tumor entities (4-12).

Orthotopically implanting intact human tumor tissue into mice leads to local and metastatic behaviour as it occurs in human patients (13-16). *In vivo* tumor imaging of orthotopic metastatic models can be achieved in real time using *in vivo* fluorescence imaging (13-17). The aim of the study was to develop an orthotopic model of peritoneal carcinomatosis of esophageal carcinoma.

Materials and Methods

Cell line. The human cell line PT1590 was isolated from a primary tumor of a patient with human esophageal adenocarcinoma at the University Medical Center Hamburg-Eppendorf as previously described.(18, 19) Cells were cultured in RPMI-1560 medium (Biochrome KG, Berlin, Germany) containing 10% fetal bovine serum (Linaris, Wertheim-Bettingen, Germany), penicillin/streptomycin (Biochrome KG, Berlin, Germany), transferrin (Sigma-Aldrich, Munich, Germany), insulin (Sigma-Aldrich), basic fibroblast growth factor (Boehringer, Mannheim, Germany) and epidermal growth factor (Boehringer). PT1590 cells were

transfected with pEGFP-N1 and pLEGFP-N1 plasmids (Clontech, Palo Alto, CA, USA) as preciously described (17).

Orthotopic injection. Ten NMRI/nu (U.S. Naval Medical Research Institute) mice were obtained from Charles River Deutschland (Sulzfeld, Germany) at 10 weeks of age and housed in the animal facility of the University Medical Center Hamburg-Eppendorf. All animal procedures were performed in accordance with a protocol approved by the Behörde für Wissenschaft und Gesundheit (Freie und Hansestadt, Hamburg, Germany). Cells were harvested after trypsinization and washed three times with medium. Mice were anesthetized with a ketamine hydrochloride (Graeub, Bern, Switzerland)/xylazine hydrochloride (Bayer, Leverkusen, Germany) mixture (12 mg/1.6 mg per ml), intraperitoneally injected at 10 ml/kg body weight. A 0.8 cm transverse incision of the skin was made in the epigastric abdomen. The abdominal muscles and the peritoneum were separated by a sharp dissection and the abdomen was opened. The great curvature of the stomach was held open with a forceps and the liver was raised to expose the abdominal esophagus. GFP-PT1590 fluorescent esophageal adenocarcinoma cells (10^6) in 20 μl HBSS suspension were injected into the submucosa of the abdominal esophagus with a Hamilton syringe unsing a 30G needle (Hamilton Bonaduz AG, Switzerland). The incision of the abdominal wall was closed using 6.0 vicryl sutures (Ethicon, Norderstedt, Germany). All procedures of the operation, as described above, were performed under a dissecting microscope (Carl Zeiss, Jena, Germany). Postoperative analgesia was achieved by novamine sulfone (1 mg/ml) in drinking water. Mice were weighed and examined for tumor development three times per week. Mice were monitored for 63 days post procedure. When the performance status of the mice decreased before day 63 due to tumor progression, the animals were sacrificed.

In vivo fluorescence imaging. Mice were monitored three times per week by non-invasive *in vivo* fluorescence imaging using a Pan-a-see-ya panoramic imaging system® (Lightools Research, Encinitas, California, USA) with fiberoptic lighting at 490 nm. No anesthesia was necessary to perform routine imaging. To improve the quality of images, mice were occasionally anesthetized with carbon dioxide for a few seconds. Images were processed for contrast and brightness using Adobe® Photoshop CS4 (San Jose, CA, USA).

At the time of sacrifice, open fluorescence imaging was performed. Primary tumor and mestastic spread was visualized, and organ localization was confirmed. After whole-body imaging, the organs and lymph nodes were dissected, removed and further examined for metastasis using fluorescence imaging. The peritoneal tumor as well as the lungs, liver, and lymph nodes were dissected and examined separately with the fluorescence imaging system.

Results

Peritoneal carcinomatosis. After orthotopic injection of human GFP-PT1590 esophageal adenocarcinoma cells, five mice (50%) developed extensive peritoneal tumor spread. However, none of these mice developed a primary tumor at the orthotopic injection site at the abdominal esophagus. Five mice (50%) did not show any signs of human GFP-PT1590 esophageal adenocarcinoma cell growth 63 days after orthotopic injection. Characteristics of the tumor-bearing mice are summarized in Table IA.

Table I. *Half of all of mice developed a peritoneal bulk tumor with diffuse spread after orthotopic injection of human PT1590 esophageal adenocarcinoma expressing GFP into the abdominal esophagus (A). All mice developed primary tumors after orthotopic implantation employing tissue fragments into the abdominal esophagus, expressing lymph node and organ metastases (B) as previously published (17).*

A. Survival, body weight and tumor progression of orthotopically injected GFP-PT1590 human esophageal cancer cells.

Mouse	Survival time (days)	Weight (g)	Day of first imaging signal	Primary tumor	Peritoneal carcinosis
1	63	28.6	34	-	+
2	63	25.7	63	-	+
3	52	34	30	-	+
4	63	25.4	/	-	-
5	63	27.2	/	-	-
6	63	25.2	63	-	+
7	63	25.6	/	-	-
8	63	23.5	/	-	-
9	63	30.2	50	-	+
10	63	25.1	/	-	-
Average	61.9	27.05	48	0%	50%

B. Survival, body weight and tumor progression of orthotopically implanted GFP-PT1590 human esophageal tumor fragments.

Mouse	Survival time (days)	Weight (g)	Primary tumor	Metastasis		Lymph node Involvement
				Liver	Lung	
1	56	36.9	+	+	+	-
2	31	29.5	+	+	+	+
3	30	30.8	+	-	+	+
4	71	25.5	+	+	+	+
5	71	29.7	+	-	-	+
Average	41.8	30.48	100%	60%	80%	80%

In all cases, the visceral as well as parietal peritoneum was affected by the carcinomatosis. Tumor spread was observed in all quadrants of the abdomen at the time of sacrifice (day 63) in all cases. A peritoneal bulk tumor was also observed in the abdominal cavity in all cases. However, the size of the actual tumor mass in the abdomen varied between animals. All mice, except mouse 3, were sacrificed at the defined end point of the study at day 63. The weight of the animals at the end point of the study ranged from 25.2 g to 34 g (Table IA). Mouse 3, which weighed 34 g, was sacrificed on day 52 due to an observed rapid weight gain and a decrease in general performance.

Whole-body fluorescence imaging was performed in real time three times per week. Fluorescence signals were first recorded in the five mice between days 30 and 63 (Figure 1B). At the time of initial detection of tumor fluorescence, there was neither a palpable abdominal tumor nor a decrease in the general performance status of the mice. Peritoneal

spread at the end point of the study was heterogeneous. Fluorescence imaging showed peritoneal carcinomatosis at different stages of tumor progression in each animal (Figure 1A, Panels A-E).

When evaluating the body weight of tumor-positive and tumor-negative mice over the time course of the study, tumor-positive mice, although showing extensive tumor growth, did not show a significant divergence in body weight (Figure 2). A slight increase in body weight was observed in tumor-positive mice during the final two weeks of the study.

Organ metastasis. At time of sacrifice, mice were imaged *in vivo* as well as at different stages of dissection. Whole-body fluorescence imaging revealed extensive abdominal spread as described above, as well as thoracic spread of the human esophageal GFP-PT1590 cells (Figure 3A). Upon performing open imaging, the peritoneal bulk tumor was removed. Various spreading lobes of the tumor were distinguished (Figure 3B). After the removal of lymph nodes and parenchymal organs, fluorescence imaging of the organs was performed separately (Figure 3C). An intensive fluorescence signal was seen in the liver, lungs, and lymph nodes. Spread to the lungs can most likely be classified as metastatic, as the thoracic and abdominal cavities are separated by the diaphragm, which was not continuously infiltrated by the tumor. Continuous or metastatic spread to the liver and lymph nodes cannot be distinguished by fluorescence imaging alone. After removal of the peritoneal bulk, tumor spread to the parietal peritoneum was visualized by fluorescence imaging (Figure 3D).

Discussion

The unique PT1590 cell line was generated at the University Medical Center Hamburg-Eppendorf from the primary tumor obtained from a patient with esophageal cancer classified as stage pT1pN1M0 according to the tumor-node-metastasis classification of the International Union Against Cancer (20). A lymph node cell line LN1590 was also obtained from the same patient. The lymph node was classified as tumor-free by routine histopathological methods, but it contained three Ber-Ep4-positive cells per approximately 10^5 lymph node cells (19), indicating micrometastatic spread in this lymph node. The tumorigenic potential of the LN1590 cell line has previously been described (21). A metastatic spreading pattern analogous to that in humans has not been achieved in mice by subcutaneous injection of these tumor cells alone (17, 21). As the treatment of esophageal carcinoma is still limited by the stage at initial diagnosis and surgical resectability, it is crucial to investigate the biology of the primary tumor as well as that of the metastatic targeting of the esophageal carcinoma. Presently, there is no effective chemotherapeutic or biological treatment for metastatic esophageal cancer.

Orthotopic models have been established for numerous tumor entities. As it would be expected in human colorectal cancer, human colon cell line suspensions injected into the cecum of nude mice led to a cecal primary tumor and also to liver metastases (6-12). Similar orthotopic mouse models have been developed for human lung carcinoma (22, 23), stomach carcinoma (24-26), bladder cancer (24, 27-29), melanoma (24, 30, 31), breast cancer (24, 32-36), as well as head and neck cancer (37). A model of squamous cell carcinoma of the cervical esophagus in rats achieved 99.5% tumor uptake with three lymphatic micrometastases in 22 rats (38). Metastasis was not detectable, although primary tumor growth was substantial in an inoculation model of squamous cell carcinoma of the esophagus (39).

It has been shown that a reliable orthotopic tumor growth is a key to achieve metastatic spread. In orthotopic models of pancreatic tumor, this can lead to a high metastatic frequency (40), with metastasis to regional and distant lymph nodes, as well as to the liver and the lungs (41). In our study, of orthotopic injection of GFP-PT1590 esophageal tumor cells, extensive peritoneal and metastatic spread was seen in 50% of the mice, although none of the animals developed primary tumor growth at the orthotopic site. The reason for lack of primary tumor growth may be the lack of tissue structure of the cell suspension, since orthotopic implantation of tumor fragments led to primary tumor growth on the esophagus (17). In this orthotopic model of esophageal tumor fragments, implanted mice developed primary tumor growth in 100% of the animals, as well as metastatic spread to the liver in 60%, and to the lung and lymph nodes in 80% of mice (17). Data of these experiments are summarized in Table IB. However, this model did not result in peritoneal carcinomatosis. In addition, metastatic spread was observed in the lungs, lymph nodes, and liver. These findings suggest that an injection model can be used as a model of peritoneal carcinosis as well as organ metastasis.

In human patients, surgical resection of esophageal carcinoma can be limited by a local 'orthotopic' tumor spread, by distant metastases to parenchymatous organs, and by local or advanced peritoneal spread. For these patients, a model of peritoneal carcinosis could be of great importance. Novel therapeutics could be tested simply by intraperitoneal injection after inoculation of esophageal carcinoma cells. Fluorescence imaging allows for highly specific visualization of peritoneal spread and distant organ metastases, such as pulmonary metastases, as shown in the present study of esophageal carcinoma. Imaging is easily obtained without the need for any contrast agent or anesthesia. The fluorescence imaging technique is simple to utilize. The short image acquisition time allows for rapid visualization without anaesthesia during scanning. Serial imaging is highly reproducible, and tumor progression can be visualized accurately.

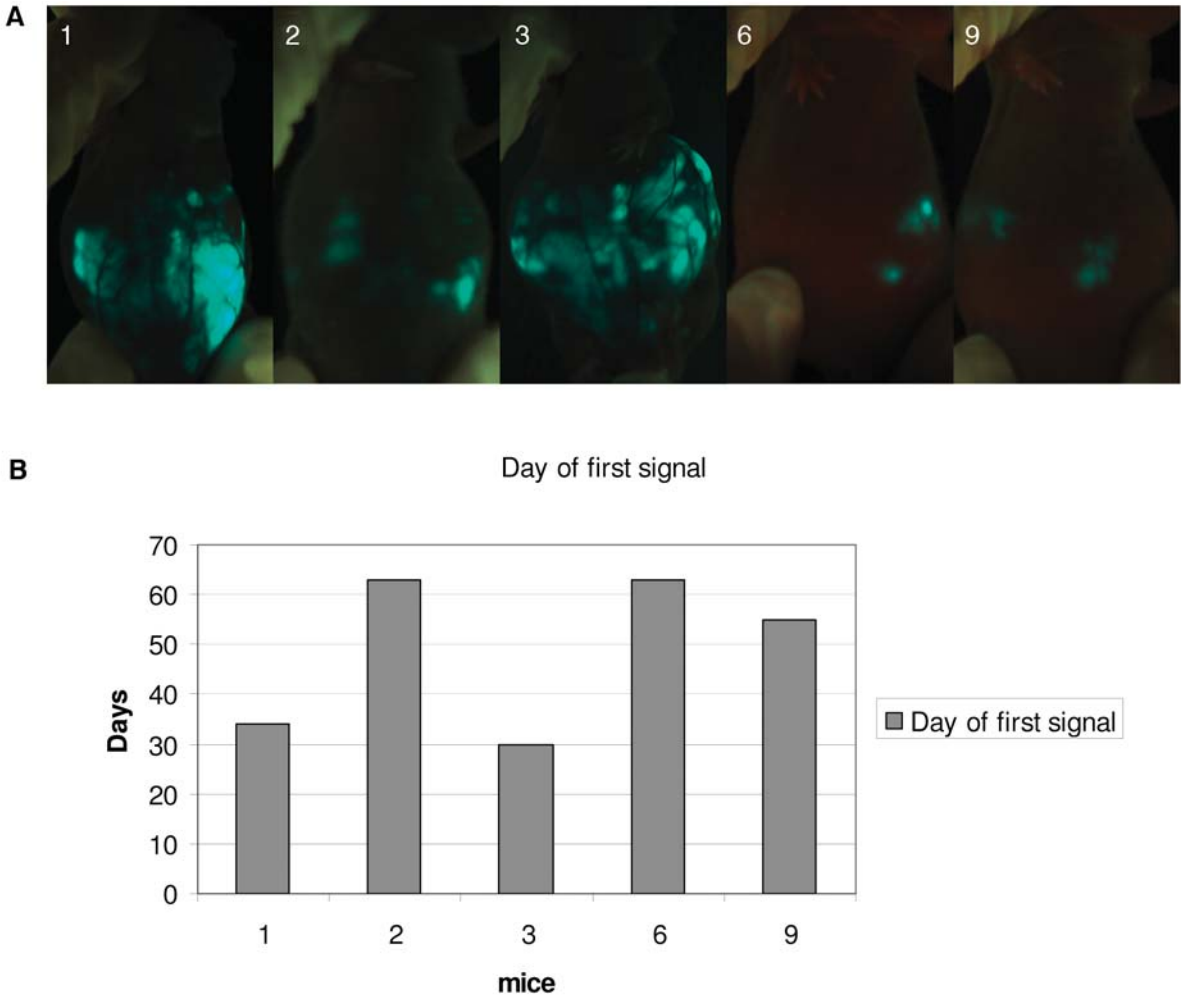

Figure 1. *Non-invasive fluorescence imaging was performed to visualize peritoneal carcinomatosis in different stages of progression (mouse 1, mouse 2, mouse 3, mouse 6, mouse 9), overall suggesting heterogenous spread. A: GFP images of each mouse. B: Day when GFP fluorescence signal was first detected.*

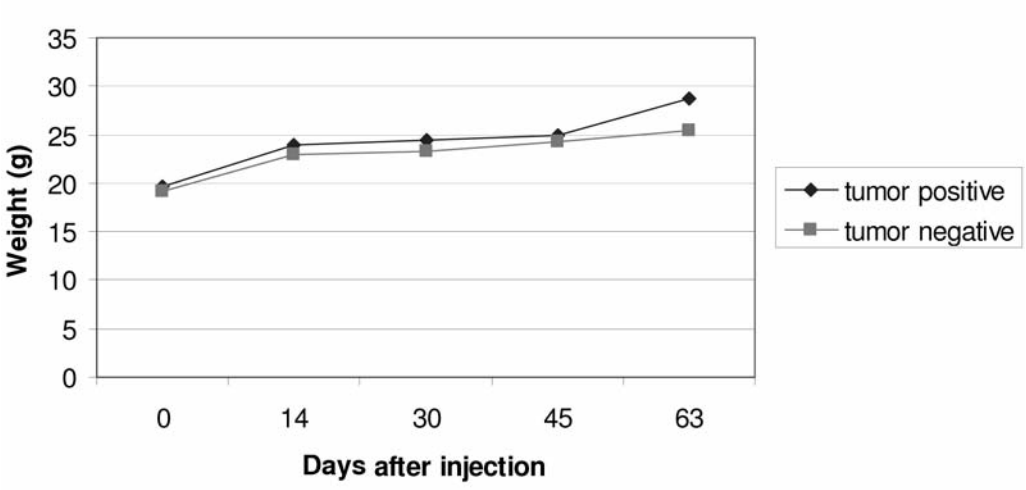

Figure 2. *Body weight of mice after orthotopic injection of human GFP-PT1590 esophageal adenocarcinoma cells.*

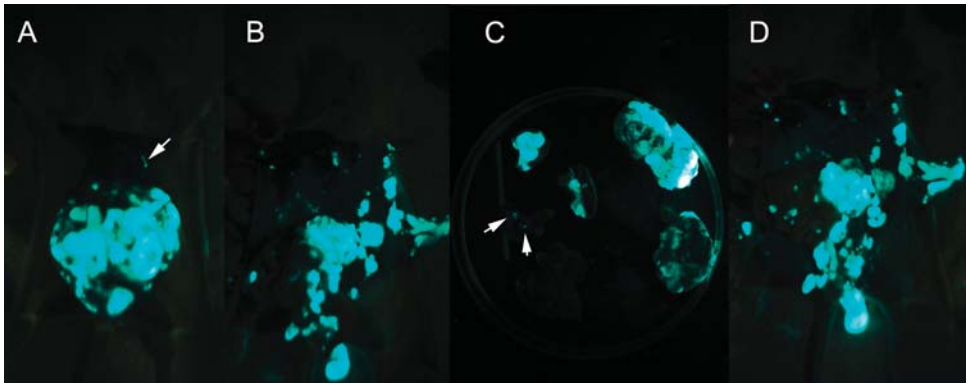

Figure 3. *Whole-body fluorescence imaging at the time of sacrifice reveals extensive abdominal and thoracic spread (indicated by the arrow) of human esophageal GFP-PT1590 cells (A). After removing peritoneal bulk tumor, which consists of various spreading lobes (B), continual tumorous spread to the parietal peritoneum was evident (D). Fluorescence imaging of the organs separately after removal shows continuous as well as metastatic spread to the liver (stronger signals), lung (weaker signals) and lymph nodes (indicated by the arrows) (C).*

Open fluorescence imaging with selective imaging of specific organs allows visualization of small metastases, especially of liver, lung, or lymph node metastases that could not be detected by whole-body imaging due to the extensive peritoneal spread.

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