RESEARCH ARTICLE

A GFP-labeled Human Colon Cancer Metastasis Model Featuring Surgical Orthotopic Implantation

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Abstract

Colorectal cancer has become a major disease threatening human health. To establish animal models that exhibit the characteristics of human colorectal cancer will not only help to study the mechanisms underlying the genesis and development effectively, but also provide ideal carriers for the screening of medicines and examining their therapeutic effects. In this study, we established a stable, colon cancer nude mouse model highly expressing green fluorescent protein (GFP) for spontaneous metastasis after surgical orthotopic implantation (SOI). GFP-labeled colon cancer models for metastasis after SOI were successfully established in all of 15 nude mice and there were no surgery-related complications or deaths. In week 3, primary tumors expressing GFP were observed in all model animals under fluoroscopy and two metastatic tumors were monitored by fluorescent imaging at the same time. The tumor volumes progressively increased with time. Seven out of 15 tumor transplanted mice died and the major causes of death were intestinal obstruction and cachexia resulting from malignant tumor growth. Eight model animals survived at the end of the experiment, 6 of which had metastases (6 cases to mesenteric lymph nodes, 4 hepatic, 2 pancreatic and 1 mediastinal lymph node). Our results indicate that our GFP-labeled colon cancer orthotopic transplantation model is useful with a high success rate; the transplanted tumors exhibit similar biological properties to human colorectal cancer, and can be used for real-time, in vivo, non-invasive and dynamic observation and analysis of the growth and metastasis of tumor cells.

Keywords: Colon cancer - fluorescence - surgical orthotopic implantation - metastasis

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Introduction

In 2008 there were 1.2 million new cases of colorectal cancer worldwide and about 608, 700 deaths caused by the cancer. Incidence of colorectal cancer is the second and the third highest among malignant tumors for men and for women, respectively (Jemal et al., 2011). Colorectal cancer has become a major disease that threatens human health. To establish animal models that exhibit the characteristics of human colorectal cancer will not only help to study the mechanisms underlying the genesis and development of colorectal cancer effectively, but also provide ideal carriers for the screening of medicines for colorectal cancer and for examining their therapeutic effects.

So far the commonly used animal models for colorectal cancer include orthotopic transplantation model, subcutaneous tumor model, gene knock-out model and chemically induced model. However, the gene knock-out and chemically induced model typically undergo substantial changes during the development of mice tumors and it is difficult to make non-invasive observations and evaluations (Alencar et al., 2005). Whereas the subcutaneous tumor model is easy to establish and convenient for observation, due to the fact that the tumor cells grow in abnormal host environment, the biological properties of the tumor changed notably (Carlomagno et al., 2006; Teicher et al., 2006). The biological properties, especially clinical-like tumor growth and metastasis, are fairly similar to human colorectal cancer, thus the SOI mouse model plays an important role in the initiation of primary tumor growth, invasion, and distant metastasis of colorectal cancer (Taketo et al., 2009).

Fluorescent imaging technique is a sensitive and fast in vivo cellular and molecular imaging method, allowing use of cells transfected with fluorescent gene or specific molecular probes and sensitive fluorescent imaging system to perform real-time, non-invasive, dynamic and in vivo observation and analysis on different gene expressions, cell surface protein markers and diseased cells. In the present study, we establish a stable, colon cancer nude mouse model highly expressing GFP for spontaneous metastasis after SOI.

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Materials and Methods

Animals and cells

BALB/C nude mice, grade SPF, were purchased from the Model Animal Research Institute of Nanjing University (laboratory animal license No. SCXK(Su) 1010-0001, animal certificate No. 2019886). All animals were male, 4-6 weeks, 20-22 grams. In this study, we used the colorectal cancer cell line HCT-116, which is an hMSH1 gene-deficient colorectal cell line. hMSH1 is one of the mismatch repair genes (MMR) and colorectal cells that are MMR gene-deficient may develop into microsatellite instability colorectal cancer. Human colon cancer cell line HCT116 was purchased from American Type Culture Collection (ATCC). HCT-116 cells were cultured in RPMI-1640 medium containing 10% FBS at 37 °C, trypsinized and then made into cell suspension at 1×10^7/L.

Construction of green fluorescent protein (GFP) pLPCX retroviral plasmids and transfection of HCT-116 cells

The GFP expression plasmid pEGFP-N3(-) was purchased from BD Biosciences (USA); the retroviral plasmid pLPCX was purchased from Clontech Laboratories (Japan). The construction and culture of GFP retroviral vector PT67-pLPCX-EGFP package cells were performed using the method reported by a previous study (Yang et al., 1999). The culture supernatant of the package cells were collected and added to cultured HCT-116 for 24-72 hours at 37 °C, 5% CO_2. The cells were then cultured in selective medium containing 800mg/L G418 (neomycin); clonal selection method was used to select GFP-positive cells, which were made into a 1.5×10^7/ml HCT-116GFP suspension.

Subcutaneous tumor nude mice model

Three nude mice were used to build the subcutaneous tumor model. The skin on the right neck of the mice was sterilized using 0.5% iodophor before 0.1 ml HCT-116GFP cell suspension was subcutaneously injected with 1ml disposable syringe. After injection, the injection site was monitored; when the GFP-labeled tumors grew to 10×10 mm in sized as observed with in vivo fluorescence imaging, colon orthotopic transplantation was performed.

1.4 Colon cancer fluorescent orthotopic transplantation

All animal experiments were completed in the animal room of the Comparative Medicine Center, Southeast University (laboratory animal use permit No. SYXK(Su) 2007-0011). The colon tumors that were transplanted and grew subcutaneously were cut into 1mm×1mm tissue chunks. Fifteen BALB/C nude mice were anesthetized via IM injection. After the mice were down, the abdomen was sterilized with 0.5% iodophor. A 1cm vertical incision was made in the left bottom abdomen of the nude mice; the skin and the peritoneum were cut open with scissors to expose the colon, which was pulled so that about 1cm was outside the peritoneum; the serous coat of the colon was cut, and then with microsurgery, 1 piece of GFP-labeled tumor tissue was implanted into the colon serosain the nude mice colon using an 8-0 surgical suture under 8x surgical microscope; the serous coat was closed before the exposed colon was placed back to the abdominal cavity and sutured to the peritoneum; a 5-0 surgical suture was used to close the abdomen. All surgical procedures were performed on a super-clean bench.

Observation of the biological properties of the GFP-labeled orthotopically transplanted colon cancer tumors

Three weeks after the tumor transplantation, the tumor biological properties in the model animal were monitored. Each week, after measuring body weight, the orthotopic tumor size in each individual animal was measured using an IFLUOR-100 small animal in vivo fluorescence imaging system, and tumor volume was calculated using Image-Pro software (Media Cybernetics, Silver Spring, MD, USA) based on the length (L) and width (W) of the tumor. In addition, a representative 1392×1040 resolution picture of each of the tumors was taken under the fluorescence imaging system at the same time.

End of experiment

When the model animals died naturally or were euthanized 7 weeks after the tumor orthotopic transplantation, the experiment was considered accomplished. Fluorescence imaging in vivo and inside the opened animal were performed; the conditions of the primary tumors and their metastasis were examined; the microvessel density on the surface of the primary tumors was measured; the primary tumors were dissected and weighed. After that, a complete necropsy procedure was performed, samples of the primary tumors, the metastatic tissues and normal colon tissues were collected.

HE staining

Tissue samples were fully fixed in 10% formalin solution and dehydrated using a Leica TP1020 automatic dryer. The samples were then embedded in paraffin and subsequently cut into 3 μm thick slices using a Leica RM2235 microtome. Slices were heated at 60 °C for one hour and then stained using routine HE staining.

Statistical analysis: statistical analysis was conducted using SPSS® 19.0 software. For comparison of quantitative data, the measurement data are presented as the mean ± standard deviation (SD), and the data were compared using analysis of variance (ANOVA).

Results

The animal models were successfully built in all 15 nude mice. All nude mice tolerated the tumor transplantation surgery, and recovered normal activity within 5-10 min after the anesthesia was terminated. There were no complications associated with anesthesia, surgery, or post-surgical infection. Tumor fluorescence was observed as early as 7 days post-transplantation. In the third week, tumors were observed under fluorescence in all model animals (100%), and no model animal died. Two model animals died in the fifth week, and 5 died in the sixth week; 8 survived till the end of the experiment. All deaths of the model animals were caused by intestinal obstruction and cachexia resulting from malignant tumor

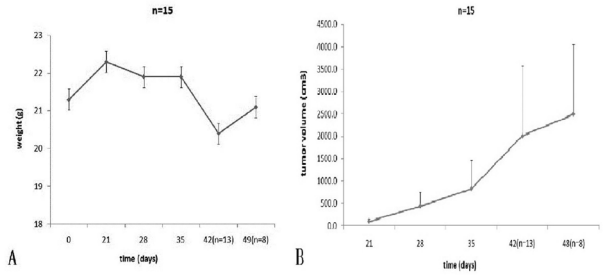

Figure 1. Animal Weight and Tumor Volume After the Implantation. (A) Animal weight was measured at different time points after the tumor implantation (error bar, SD; F=2.98, P=0.042). (B) Tumor volume was measured at different time points after the implantation (error bar, SD; F=13.902, P=0.005)

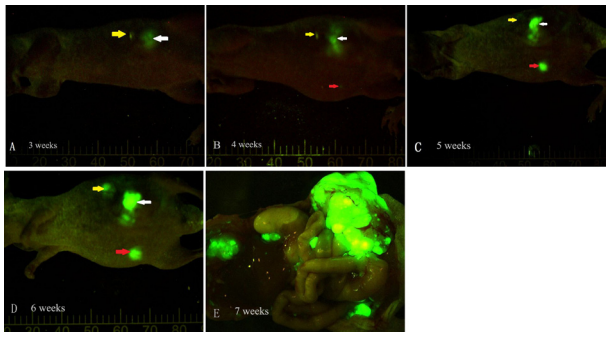

Figure 2. Fluorescent Images Showing Distant Tumor Growth in the SOI Models were True Time-dependent Metastases. Tumor fluorescence could be observed at 3 weeks (A), 4 weeks (B), 5 weeks (C), 6 weeks (D) and 7 weeks after the implantation (E). The implanted tumor was indicated by white arrows, liver metastasis was indicated by yellow arrows and lymph node metastasis was indicated by red arrows

growth, and no accidental death occurred.

At the early stage after the orthotopic transplantation, the weight of model animals showed an increasing tendency; yet as the tumor grew, the mice demonstrated cachexia and weight loss (Figure 1A). The volume of orthotopically transplanted tumors increased with time and were easily measured under fluorescence imaging system. Figure 1B shows the volume of orthotopically transplanted tumors measured by Image-Pro5.1 under the global fluorescence imaging system.

From the third week after the transplantation, tumor metastasis could be observed under the fluorescence imaging system and allowed monitoring of tumor progression in a single animal by sequential imaging analysis at different time (Figure 2). The microvessel density of the orthotopic tumors and metastasis in the transplantation model were studied at the end of the experiment. At the end of the experiment, metastasis occurred in 6 out 8 survived model animals, among which there were 6 cases of metastasis to mesenteric lymph nodes, 4 cases of hepatic metastasis, 2 cases of pancreatic metastasis, 1 case of lumbar lymph node metastasis and 1 case of metastasis to mediastinal lymph node (Table 1). The average microvessel density of the tumors in the model animals was 0.67 mm/mm^3 (Figure 3). The pathological results showed that the primary and metastatic tumors were both poorly differentiated adenocarcinomas (Figure 4).

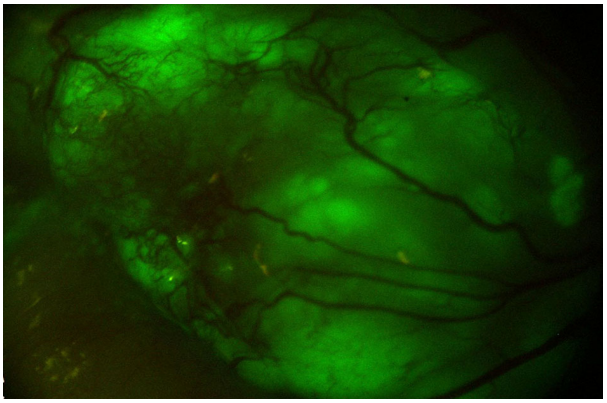

Figure 3. Fluorescent Imaging of Angiogenesis in Orthotopic Primary Tumor (×8). The nonluminous angiogenic blood vessels appear as sharply defined dark networks against the bright background

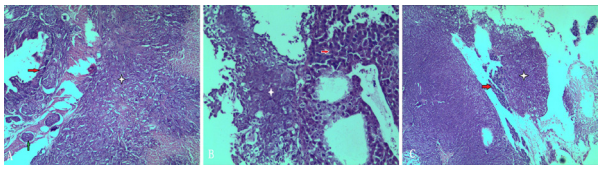

Figure 4. Histology Confirmed the Tumor Metastasis. HE staining showed tumor metastasis in the implanting site (A), liver (B) and pancreas (C). The red arrows show the normal tissue and the stars show the metastasis. The green arrow shows the vein tumor thrombus

Table 1. Tumor Metastasis at the End of the Experiment

Animal #(n=8)	Sites of metastasis
1	mesenteric lymph nodes, liver
3	mesenteric lymph nodes
4	mesenteric lymph nodes, liver
8	mesenteric lymph nodes, liver, pancreas
9	mesenteric lymph nodes
14	mesenteric lymph nodes, liver, pancreas, mediastinal lymph nodes

Discussion

Our study designed a fluorescent colon cancer orthotopic transplantation model, in which HCT-116 cells transfected with GFP were directly transplanted into the serosa of the colon and the colon was secured to the corresponding peritoneum after transplantation. After 3 weeks, tumor growth was observed under fluorescence in all transplantation models. There was no complication related to the transplantation surgery, and the death rate of the model animals did not increase. In addition, as the tumor growth time prolonged, the transplanted tumor exhibited corresponding invasive behavior. Meanwhile, with these fluorescent tools, the combination of green fluorescent protein-based imaging in SOI mice models enables real-time, non-invasive and dynamic observation and analysis of tumors and metastasis in host organs.

It has become evident that the main problem in the treatment of CRC is not so much eradication of the primary tumor, but rather the formation of incurable metastases. Mortality is particularly associated with the occurrence of metastases in the liver (Bouchahda et al., 2011; Lehmann et al., 2012; Spelt et al., 2012). Although multiple gene

knock-out and chemically induced colorectal cancer models have been established, most of these tumor models do not exhibit invasive behavior of colorectal cancer, such as metastasis to liver, lung and lymph nodes (Sun et al., 1999; Heijstek et al., 2005; Taketo et al., 2009). In addition, genetically modified mice show tumor dissemination at significantly longer time periods (1.5 to 2 years), develop tumors in the small bowel rather than in the large bowel (Kobaek-Larsen et al., 2000).

On the other hand, Jin (Jin et al., 2009; Jin et al. 2011) had established the orthotopic models of subcutaneous transplantation with HT-29 cell lines and colostomy implantation model with LoVo cell lines. However, they did not detect any liver metastasis in these models, which is the most important characteristic seen clinically in human colonic carcinomas. These results have been reported by some other authors (Kubota et al., 1994; Pocard et al., 1996; Bibby et al., 2004). Currently the orthotopic transplantation model is considered as an ideal model to study tumor metastasis and to analyze the treatment effect of drugs (Taketo, 2006). In this study, the results showed that after HCT-116 cells transfected with GFP were orthotopically transplanted to nude mouse colon, metastasis to liver, pancreas and lymph nodes, but not to lung, was observed.

Cespedes et al. (2007) orthotopically injected human colon cancer cells to the nude mouse appendix using micro-suction tube, and the result showed that relatively high level of metastasis to lymph nodes, liver, lung and peritoneum occurred in the formed tumor. However, their work did not perform a consecutive analysis with the primary tumor and metastasis, and they reported that 50% of their model had generated tumor foci in the lung - a phenomenon that is not common in the colorectal cancer. Instead of injecting cell suspensions into the orthotopic site, we have used microsurgical technology to transplant HCT-116-GFP orthotopically. Our study showed that tumor metastasis could be observed under the fluorescence imaging system and allowed monitoring of tumor progression in a single animal by sequential imaging analysis at different time. It is uniquely useful for innovative drug discovery and mechanism studies and serve as a bridge linking preclinical and clinical drug development.

We have established a GFP-labeled colon cancer orthotopic transplantation model with high survival rate. The transplanted tumors exhibit similar invasive biological properties as in human colorectal cancer, and can be used for real-time, in vivo, non-invasive and dynamic observation and analysis of the growth and the metastasis of transfected tumor cells. Futher studies with this model are clearly warranted.

Acknowledgements

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