A Nude Mouse Model of Massive Liver and Lymph Node Metastasis of Human Colon Cancer

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Abstract. Liver and lymph nodes metastasis are the main causes of treatment failure for advanced colon cancer. However, currently-available animal models of human colon cancer do not demonstrate sufficient metastasis to represent highly malignant colon cancer that extensively metastasizes to these sites. A liver metastasis from a patient with highly malignant, poorly differentiated adenocarcinoma of the colon was established in nude mice by surgical orthotopic implantation to the mouse colon. The human origin of the tumor growing in nude mice was confirmed by in situ hybridization of human DNA. After 20 passages from the first implantation, massive liver and lymph nodes metastasis, occurred in 100% of the transplanted animals. Lymph nodes metastasis were found at the sites of lymph node drainage of the liver: celiac, portal and mediastinal lymph nodes. However no mesenteric and retroperitoneal nodes or lung tissue metastasis were observed. Our data suggest that the mediasinal, celiac and hepatic lymph nodes metastases are derived from the liver metastasis, confirming the concept of metastasis of metastases or “remetastasis” of colon cancer.

There are 131,000 new cases of colorectal cancer diagnosed per year in the United States [1]. Lymph-node and liver metastasis are the two major obstacles for treatment of colon cancer. At presentation with colorectal carcinoma, 26% to 44% of patients have positive lymph nodes [2]. The survival time of these patients is related to the number of lymph nodes involved [3]. Approximately 50% of patients with colorectal carcinoma develop recurrence within five years after treatment of their primary colorectal cancer. The liver is the site of recurrence in 40% to 80% of the cases [4-8].

The lymphatic system is an important route of spread of hepatic metastatic disease to extrahepatic sites. Although portal and celiac nodes are commonly evaluated both pre- and intraoperatively in patients considered for resection, cephalad sites of lymphatic drainage of the liver represent a more occult problem [9]. Since the spread of colorectal cancer may be initially limited to the liver, surgical cure of distant disease is possible, with even repeated resections for recurrent lesions [10]. However the possibility of lymph-node metastasis from liver metastasis or “remetastasis” renders surgical cure less likely. Hughes [11] reported that, although resection of extrahepatic disease may in some cases lead to long-term survival, remetastasis to the hepatic or celiac nodes leads to significantly decreased long-term survival, even with aggressive operative intervention. However the occurrence of remetastasis is not a fully accepted concept [3].

Lymphatic spread of hepatic lesions is usually via intrabdominal lymphatic routes. With increased survival of patients with hepatic metastasis from colon cancer, mediastinal lymphatic spread of metastatic liver disease from colon cancer is becoming more common.

However, there is a lack of effective treatment and accurate diagnosis of metastatic lymph-nodes originating from liver metastasis of colon cancer. Therefore clinically-relevant animal models for the study and development of new therapeutics and diagnostic methods for lymph node metastases of colon cancer are greatly needed.

Surgical orthotopic implantation (SOI), which involves the orthotopic transplantation of histologically intact tumor fragments, has allowed the development of models of human cancer in nude mice that demonstrate the variety of clinical behavior that occurs in human patients [12-20]. Metastatic models developed with SOI exhibiting patients-like metastasis include colon cancer [13] lung cancer [14], bladder cancer [15], pancreatic cancer [16], prostate cancer [17], ovarian cancer [18], and stomach cancer [20].

We report here an SOI model of highly malignant colon cancer, derived from a patient’s liver metastasis that metastasizes diffusely in the liver parenchyma and in lymph nodes in 100% of the transplanted animals.
Materials and Methods

Animals. Male and female athymic BALB/c nude mice between 4 to 6 weeks of age were used in this study. The animals were bred and maintained in a HEPA-filibrated environment. Cages, food and bedding were sterilized by autoclaving. The breeding pairs were obtained from Charles River Laboratories (Wilmington, MA). The animal diets were obtained from Harlan Teklad (Madison, WI). All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

Surgical specimen. The fresh surgical specimen was obtained from a resected liver metastasis of a patient with colon cancer at the Department of Surgery, School of Medicine, University of California, San Diego. The surgical specimen was kept at 4°C in Earl's minimal essential medium (MEM). The specimen was transplanted using SOI in nude mice within 24 hours of surgery.

Before implantation, specimens were washed twice with antibiotic-containing Earl's MEM, at least 10 minutes each time to prevent possible contamination and infection. After necrotic tissue and non-cancerous tissue of the specimen were removed, the remaining cancerous tissue was divided into small pieces approximately 1 mm in diameter. The tumor, termed AC3488, was derived from a 46-year-old man with poorly differentiated adenocarcinoma of the colon, Stage IV with liver metastasis. The specimen was obtained from the right lobe of the liver after resection. The patient died of metastasis in the liver seven months after operation.

Implantation procedure. For surgical orthotopic implantation (SOI) to the colon [13], nude mice were anesthetized with isoflurane (Forane) inhalation. The abdomen was sterilized with iodine and alcohol swabs. A small midline incision was made and colocolic part of the intestine was exteriorized. The serosa of the colon was removed and 1-2 pieces of 1 mm3 size tumor fragments per mouse were implanted. An 8-0 nylon surgical suture was used to penetrate these small tumor pieces and suture them on the wall of the intestine. The intestine was returned to the abdominal cavity and the abdominal wall was closed with 6-0 silk surgical sutures. Animals were kept in a barrier facility under HEPA filtration.

Evaluation of growth and metastasis. Mice were sacrificed if they developed signs of distress. At autopsy, the liver, lymph nodes, lung, kidney, spleen and other organs were resected and processed for routine gross and microscopic examination. Metastasis was considered to have occurred if at least one microscopic metastatic lesion was found in any of the mice.

Human DNA detected by in situ hybridization. The human origin of the tumor growing in nude mice was confirmed by in situ hybridization of human DNA. A human DNA probe (PS080-8.5) and hybridization kit (S1346-KIT) were purchased from Oncor (Gaithersburg, MD). Fresh tumor tissues were fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin. Four sections were layered on silanized slides which were assayed for human total DNA by in situ hybridization. Briefly, tumor tissue sections on slides were baked at 65°C for 14 hours, deparaffinized in xylene, hydrated in graded ethanol, and placed in preheated protein digestion solution at 45°C for 20 minutes. Slides were dehydrated in graded ethanol for hybridization.

Probe preparation: the human DNA probe was prewarmed at 37°C for 5 minutes, denatured for 5 minutes in a 70°C water bath for 5 minutes, and quickly chilled on ice and centrifuged for 2-3 seconds. Slide preparation: slides were placed in 70% formalin/2 x SSC at 37°C for 2 minutes and dehydrated in cold (20°C) graded ethanol for 10 minutes. Hybridization procedure: DNA probe was added to slides which were prewarmed to 37°C. Glass coverslips were sealed with rubber cement.

The slides were transferred to 50% formamide/2 x SSC and 1 x PBS. The slides were then incubated with FITC-labeled Avidin for 25 minutes at 37°C. Cells were counterstained with propidium iodide (0.3 µg/ml in antibody). Slides were observed under a microscope equipped with epifluorescence [31].

Immunohistochemical study. CEA expression was determined by using rabbit anti-human CEA antibody (C-2331 lot 554853, Sigma, MO, USA). Human tumor tissue obtained from mice was fixed in formalin and embedded in paraffin. 5 µm sections were assayed for CEA expression by immunoperoxidase staining using the avidin-biotin complex method (Immunohistochemical Staining Kit, Product No. 00-601, Biomedica, CA, USA).

Results

AC3488 is a poorly differentiated adenocarcinoma of the colon, Dukes' classification D. The surgical specimen was obtained from a liver metastasis. The original tumor fragments from the patient were implanted at three sites in the nude mice: colon, liver and subcutis. After a latency of 3-4 months, the tumor grew in all implanted sites without any sign of distant metastases. After 10 generations of orthotopic passages (colon to colon) for almost two years in nude mice AC3488 developed multi-organ metastases: massive liver metastasis in 100% of the animals, spleen metastasis in 11% of the animals and hepatic lymph nodes metastases in 43% of the animals [22]. However, no mediastinal and celiac lymph node involvement was observed between passages 10 and 20.

Local tumor growth in the colon was slow and the size of tumor was small in comparison to the disseminated liver (Figure 1). Liver metastases occurred on the tenth day after transplantation, when small visible metastatic colonies were seen in all lobes of the liver. Extensive liver metastasis caused edema around the liver tissue which resulted in the expansion of the liver up to 6-10 times its normal weight (Figure 2).

After passage 20, AC3488 developed spontaneous portal, celiac, and mediastinal lymph node dissemination in 100% of the animals without any mesenteric or retroperitoneal lymph node involvement [Table II]. During orthotopic passage 20, 100% of the animals had diffuse liver metastasis, the spleen was involved with metastasis in 50% of the animals, the kidney was involved in 10% of the animals and in 10% of the animals, adrenal gland metastasis was observed in addition to the lymph node metastasis. No lung metastasis was observed in any of the animals [Table II]. Table III compares metastatic sites before and after the 20th passage.

In the murine model, the abdominal lymphatics are divided into anterior and posterior routes draining the liver including the hepatic hilum lymph nodes and celiac lymph nodes as well as through the cephalad route to the posterior mediastinal lymph nodes. Before the 20th passage the site of lymph node involvement was limited to the hepatic hilum lymph nodes draining the liver. However, after the 20th passage all other routes of lymph node drainage from the liver were involved by massive metastasis (Figure 3, A.B.C). Multiple lymph node
metastasis was observed as early as after 19 days after tumor implantation.

Histological characteristics. Histological findings of AC3488 cells demonstrated poorly differentiated adenocarcinoma at all sites (Figure 4). Histological examination of the liver revealed massive liver and blood vessel metastatic involvement and tissue edema surrounding the metastases (Figure 5). Lymph nodes, at histological examination, appeared massively infiltrated by metastatic cells (Figure 6).

Human DNA in situ hybridization. Human DNA in situ hybridization was positive in all AC3488 tumor cells and indicated the human origin of AC3488 tumor grown in nude mice (Figure 7).

CEA staining. CEA Immunohistochecmical staining was not detected in the original tumor or in the AC3488 tumor cells.

Survival. Before the 20th passage, all animals died by day-40 with a median survival of 26 days. After the 20th passage, rapid progression of AC3488 lead to the death of all animals by day-30 with a median survival of 22 days. Figure 8 shows the difference in survival time before and after the 20th passage.

Discussion

Many studies have demonstrated the importance of liver metastasis for the prognosis of patients with subsequent metastatic colon cancer [2-4, 11]. However, the presence of liver metastasis together with metastasis of lymph nodes draining the liver, including their number and their sites, can influence survival and treatment strategies.

Vines, over 55 years ago, discussed the possibility of metastasis from metastases or "remetastasis" [23]. There are significant clinical implications of the occurrence of remetastasis from liver metastasis to the lymph nodes.
Figure 3. A) Routes of lymph nodes draining the liver: mediastinal I A (white arrows); hepatic hilum I B (white arrow); and celiac I C (white arrow). All nodes were massively involved by metastasis (Bar = 1 cm).
draining the liver. Hughes et al [11], in a multi-institution review of 859 patients who had undergone liver resection for metastasis from colon cancer, reported that patients with metastasis of hepatic and celiac lymph nodes draining the liver from colon cancer have a significantly decreased survival despite node dissection and hepatic resection. Extrahepatic disease is now considered a contraindication to liver resection.
Figure 8. Survival curve of tumor-bearing animals after and before 20th passage shows decreasing survival after the 20th passage. Decreased survival is concomitant with massive metastatic involvement of lymph nodes draining the liver after the 20th passage.

Table I. Sites of metastasis of human colon cancer AC3488 in nude mice.

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<tr>
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* Suprarenal gland

Vetto and August [9, 24] reported that liver metastasis from colon cancer is capable of remetastasizing to the lymph nodes draining the liver. This event is considered an unrecognized rather than a rare one [9]. In man there are at least four possible routes of remetastasis via lymph draining the liver [9, 24]: 1) From the falciform ligament and left lateral lobe to the mediastinal lymph nodes. 2) Through the right hemidiaphragm, esophageal hiatus and caval foramen to
the mediastinal lymph nodes. 3) From the superior border of lesser omentum to the left gastric lymph nodes. 4) From the hilum to the hepatic nodes, celiac nodes, and into the thoracic duct (Figure 9).

In murine models there are at least three routes of lymphatic drainage of the liver as described by Hebel and Lambert [25,26]: 1) Abdominal, anterior, which is related to the hepatic artery and to the portal vein. 2) Abdominal, posterior, from its origin in the hilum of the liver it passes across the mesentery to the anterior surface of the abdominal part of the esophagus. These two ducts unite to form a single collecting duct which follows the hepatic artery on its lower border and from here discharges lymph to the celiac nodes and subsequently to the cisterna chyli. Lymph glands are found around each of these collecting ducts. 3) The cephalad routes draining the surface of the liver from the left and right sides to the posterior mediastinal lymph nodes (Figure 10).

In the model described in this report all 3 routes of lymph node drainage of the liver, hilum of the liver or portal, celiac as well as mediastinal routes were massively involved by metastasis (Figure 3, A.B.C).

In this highly metastatic colon cancer model which resembles the natural history of highly metastatic colon cancer in human beings, remetastasis from the liver metastasis of colon cancer to the lymph nodes draining the liver seems to occur.

Mediastinal and abdominal lymph node metastasis, including number and size rapidly increase after the appearance of hepatic metastasis in the model thereby suggesting their hepatic origin [Table IV].

After the 20th passage, when massive lymph node metastasis in sites draining the liver appeared, the median survival time of the animals decreased from 26 to 22 days suggesting that massive lymph node metastasis decreases survival time. This suggests that prognostic factors for patients with liver metastasis from colon cancer can be related to the sites and the number of lymph nodes draining the liver analogous to how lymph node draining the primary colon cancer are a prognostic factor for primary colon cancer. With new therapeutic approaches the survival time of patients with metastatic colon cancer is increasing. From results with this experimental model, we predict that increasing the survival
time of patients with advanced metastatic liver from colon cancer should increase metastatic involvement of abdominal and mediastinal lymph nodes. The model should be highly useful to understand and design new therapeutics for this highly advanced stage of colon cancer.

References


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