



Establishment of a stable hepatic metastasis mouse model of murine colorectal cancer by microsurgical orthotopic implantation

Zhiyun Cao^{1,2}, Zhideng Zhang³, Liangpu Zheng^{1,2}, Jiumao Lin^{1,2}, Jun Peng^{1,2}, Wei Lin^{1,2}

¹Academy of Integrative Medicine of Fujian University of Traditional Chinese Medicine, Fuzhou 350122, China; ²Fujian Key Laboratory of Integrative Medicine on Geriatrics, Fujian University of Traditional Chinese Medicine, Fuzhou 350122, China; ³Technology Center of Fuzhou Customs District, Fuzhou 350001, China

Contributions: (I) Conception and design: W Lin, J Peng; (II) Administrative support: J Lin, J Peng, W Lin; (III) Provision of study materials or patients: W Lin; (IV) Collection and assembly of data: Z Cao, Z Zhang, L Zheng; (V) Data analysis and interpretation: Z Cao, W Lin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Wei Lin. Associate researcher, Academy of Integrative Medicine Biomedical Research Center, Fujian University of Traditional Chinese Medicine, 1 Huatuo Road, Minhou Shangjie, Fuzhou 350122, China. Email: linwei311616@126.com.

Background: Liver metastasis is a common cause of death from colorectal cancer (CRC). In this paper we developed a liver metastasis mouse model by microsurgical orthotopic implantation (MSOI) to illuminate the CRC progression with an eye toward developing effective drug treatment.

Methods: Murine colon carcinoma CT-26 cells were cultured and then injected to male BALB/c athymic nude mice right flank to generate subcutaneous implantation tumor with 2×10^7 CT-26 cell suspension in DMEM. Tumor tissue at an average size of 1 cm³ was injected into another nude mice right flank with 20-gauge inoculating needle. Between fourth and sixth generations, tumor tissue sewn into the cecal surface establishes orthotopic transplanted CRC model by MSOI. Then on the 7th, 14th, 21st and 35th day, body weight, abdomen circumference, volume of ascites and local tumor weight were observed and weighed. On the 21st day and 35th day, local tumor rate was calculated, and metastatic tumors of other organs were observed. Tumor tissue was stained by HE for pathologic analysis.

Results: On the 35th day, body weight and abdomen circumference of the model group were significantly higher than the control group ($P < 0.01$). Local tumor weight increased rapidly from the 21st d to the 35th d ($P < 0.01$), and take rate was high (100%). Metastatic tumor appeared only in liver on the 21st day and then invaded to liver, stomach, retroperitoneal lymph node and abdominal wall on the 35th day. The metastatic rate of liver tumor respectively was 83.3% and 100% on the 21st day and 35th day, but liver function remained normal. Pathologic analysis showed that colorectal tumor invaded the normal tissue of liver, abdominal wall and stomach.

Conclusions: A stable hepatic metastasis mouse model of murine CRC was established by MSOI.

Keywords: Colorectal cancer (CRC); hepatic metastasis; mouse model; microsurgical orthotopic implantation (MSOI)

Submitted Sep 25, 2019. Accepted for publication Jan 15, 2020.

doi: 10.21037/tcr.2020.03.61

View this article at: <http://dx.doi.org/10.21037/tcr.2020.03.61>

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors today. The incidence and fatality rates of CRC have increased rapidly over recent years, likely attributable to dietary changes in both eastern and western countries (1,2). The most common cause of death from CRC is distant metastasis and invasion into other organs, such as liver, lung, bone and brain. Among distant metastatic sites, the liver is the most frequently affected organ, making hepatic metastasis one of the most common causes of death attributable to primary CRC (3,4). Some clinical reports have shown that, according to postmortem examination results, about 60% to 70% of patients in the late stage of CRC develop liver metastasis. Their median survival term is approximately 5 to 6 months, and five years survival rate drops to zero if these patients do not receive medical treatment (5). If, however, liver metastasis can be inhibited, the prognosis of CRC would be more favorable than it is currently. It is necessary to establish a liver metastasis of colon cancer mouse model so that researchers are able to illuminate the disease progression and to develop effective therapies and drugs.

Several techniques for building a metastatic CRC mouse model have been described in recent literature. Methods investigated have included orthotopic cell injection, spontaneous metastasis model, intraabdominal spleen transplantation (spleen removal group and spleen reservation group), rectum transplantation, portal injection and tail vein injection. However, spontaneous metastasis models have yielded uneven results. Spleen transplantation and tail vein injection to establish metastatic CRC mouse model were first selected by our team because of the ease of operation; however, liver metastasis appeared too early among established mouse models to be suitable for drug research. Orthotopic cell injection is an effective technique for developing modified mouse models of CRC metastasis. However, the actual volume of cells injected is not easy controlled since the mesenteric interspace is small, usually resulting in local tumor volume and dissemination sites being too widely variable. Shortcomings of other techniques include not producing metastasis at all or producing only singular metastatic foci (6,7).

Surgical orthotopic implantation (SOI) is an efficient technique to establish a viable metastatic animal model. A number of studies reported that metastatic xenograft mouse models were established with orthotopic patient-derived tissue, such as melanoma, breast cancer or hepatoma (8-10).

Building on the success in principle of SOI, we achieved modest improvement using microscopic surgical techniques [microsurgical orthotopic implantation (MSOI)] to develop a liver metastasis of orthotopic CRC mouse model with mouse-derived tissue consisting of transformed CT-26 cells.

Methods

Materials and reagents

Cell culture medium Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were both purchased from Invitrogen (Thermo Fisher Scientific, MA, USA). Penicillin 100 Units/mL streptomycin 100 µg/mL and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were also purchased from Invitrogen.

Cell culture

Murine colon carcinoma CT-26 cells were obtained from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM containing 10% (v/v) FBS in 5% CO₂ at 37 °C. The cells were subcultured at 80% to 90% confluency.

Animals

BALB/c mice (with an initial body weight of 20–22 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with controlled temperature (22 °C) and humidity and a 12-hour light/dark cycle. Food and water were provided ad libitum throughout the experiment. All animal treatments were performed strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

Subcutaneous tumor implantation in nude mice

Mice were subcutaneously injected in the right flank with 2×10^7 CT-26 cell suspension in DMEM. After 16 days of xenograft implantation, tumors were implanted to different nude mice by 20-gauge inoculating needle at an average size of 1 cm³. Tumor growth among four generations would be suitable for MSOI into the cecum surface.

Metastasis of CRC mouse model with MSOI (11)

Subcutaneous tumors were excised, and then centrally located tumor tissue was cut into pieces (volume about 1 mm^3) and placed in normal saline for later use. Mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (Abbott Laboratories, 70 mg/kg weight) and fixed in the prone position on the operating table. After the hair was shaved with an electric razor, a tiny incision was made in the skin with surgical scissors. The blind end of the cecum was elevated out with forceps and placed on a sterile gauze alongside the incision. The operating table was moved under the microscope, and microsurgical techniques were used. With stretching and leveling of the blind end of the cecum using smooth forceps, a small rent was made in the serous membrane to create a double-walled pouch in the wall of the cecum. A piece of tumor tissue derived from CT-26 was sutured into the pouch with a single Maxon 11-0 suture (*Figure S1*). After tumor implantation, the abdominal wall was closed in two layers with Dexon 5-0. Food and water were given ad libitum. Following recovery from cecal implantation of the colon tumor, the mice were randomly assigned between model and control groups ($n=18$ per group). At 7, 14, 21 and 35 d from tumor implantation, the animals were sacrificed as follows: three mice on the 7th and 14th days, six mice on the 21st and 35th days. Subsequently, the tumor tissue was removed and weighed. Then we recorded all macroscopic tumor deposits or abnormalities in the liver, and the number of liver metastases was calculated. All neoplasms were identified with hematoxylin and eosin (HE) staining.

HE staining

Tissues were fixed with 10% buffered formalin for 24 h. Samples were then paraffin-embedded, sectioned and stained with HE. Histopathological changes were observed under a light microscope.

Statistical analysis

All data are the means of six mice, analyzed using SPSS Package for Windows (Version 18). Statistical data analysis was performed using Student's *t*-test. Differences where $P<0.05$ were considered statistically significant.

Results

BALB/c mice growth after transplanted CT-26 tumor tissue by MSOI

The BALB/c mice showed normal activity in the first 21 d after MSOI of murine CT-26 colon carcinoma tissue. Gradually over the ensuing 14 days, enlarged abdomen, slow-going, weight gain, food intake decrease and hair drying. There was no difference in body weight between control and model groups in the first 21 days. After 28 days the abdomen began to enlarge and body weight rapidly increased, reaching 39.5 ± 6.2 g on the 35th day. Compared with control group, the body weight of model group increased 14.9 g (*Figure 1A,B*, $P<0.01$). The abdomen circumference was about 4.3 ± 0.8 cm in model group but only 2.8 ± 0.5 cm in control group on the 35th day (*Figure 1C,D*, $P<0.01$). The volume of ascites of the model group mice was about 3.9 ± 0.7 mL.

Local tumor growth and take rates on the 21st day and 35th day

Implanted tissue began to form solid tumor on the 7th day with the volume of tumor about $2 \text{ mm} \times 1 \text{ mm}$. On the 14th day, local tumor volume had doubled. After 14 days, local tumor began increasing in volume more rapidly. On the 21st and 35th day, the local tumor weights were 0.5 ± 0.2 and 1.1 ± 0.4 g respectively (*Figure 2*, $P<0.01$). The murine colon carcinoma cell line CT-26 showed high take rates. Among model group mice, five out of six mice generated local tumor (83.3%) on the 21st day, and all six mice (100%) were observed to have local tumor growth on the 35th day (*Table 1*). The spleen volume of the model group was twice that of the control group, and the weight of spleen in the model group had increased 0.09 g more than the control group (*Figure 3*, $P<0.01$).

Tumor dissemination sites on the 21st day and 35th day

After 21 days, liver metastatic foci were observed, but there was no ascites. Some nodules appeared in liver lobe locations but no in hepatic lobules. The size of metastatic nodules ranged from needle-tip to $2 \text{ mm} \times 1 \text{ mm}$, and they were scattered over the surface of the large liver lobe and hepatic lobules. Liver color and shape remained normal (*Figure 4A*). CT-26-generated metastatic tumor in liver was

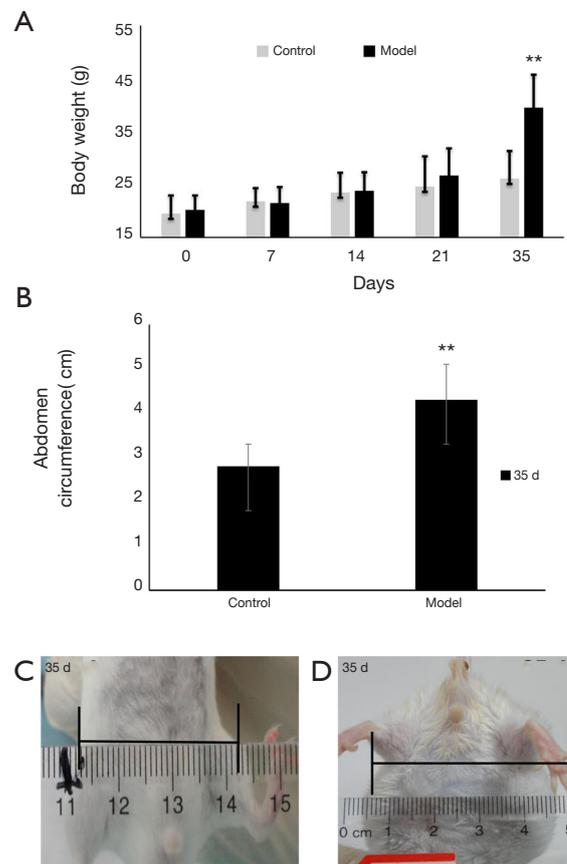


Figure 1 Body weight and abdomen circumference of orthotopic CRC mouse model. After mouse model was established by MSOI, on the 7th, 14th, 21st and 35th days, control and model group mice were weighed, and abdomen circumference on the 35th day measured. Body weight differed little between model and control groups before the 21st day but was significantly increased on the 35th day. (A) Body weight of control and model group on the 0, 7th, 14th, 21th, 35th days. Model group body weight sharply increased on the 35th days. Data presented as the mean \pm standard deviation from six individual mice in each group. **, $P < 0.01$ vs. controls. (B) Abdominal circumference of model group was significantly greater than control group. Data presented as the mean \pm standard deviation from six individual mice in each group. **, $P < 0.01$ vs. controls. (C,D) Abdomen images are representative. CRC, colorectal cancer; MSOI, microsurgical orthotopic implantation.

present in about five out of six mice (83%) on the 21st day (Table 1). After 28 days, model mice begin to produce ascites with subsequent gradual tumor dissemination to other organs. On the 35th day there were multiple organ metastases, including to liver, stomach and abdominal wall. Liver lobe and hepatic lobules of mice that generated hepatic metastasis were covered with tumor nodules of different sizes, the largest one about peanut-sized or larger (5 mm \times 5 mm), the smallest nodules about rice-sized in a diffuse distribution, with almost no normal liver tissue remaining. Liver color and shape were abnormal compared control group (Figure 4A). CT-26-generated metastatic

tumor in liver was about six out of six (100%) on the 35th day (Table 1). Liver function values (AST and ALT, Table S1) of the model group on the 21st day and the 35th day were all normal compared with control group. On the 35th day the metastatic sites included retroperitoneal lymph node, abdominal wall and stomach. Metastatic rates were six out of six (100%), three out of six (50%) and six out of six (100%), respectively (Figure 4B,C, Table 1).

Local and metastatic tumor pathologic analysis

Pathological changes of local tumor and tumor

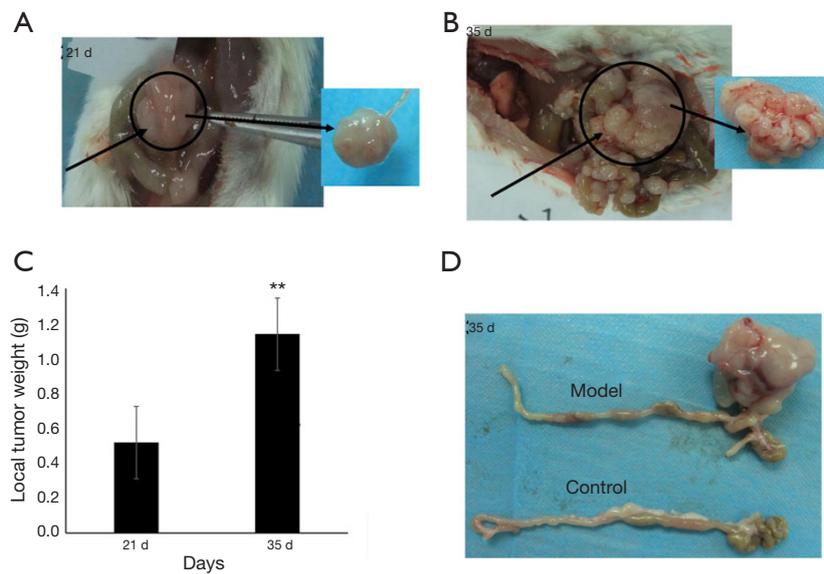


Figure 2 Local tumor weight of orthotopic CRC mouse model. After mouse model was established by MSOI, on the 21st day or the 35th day, mice were sacrificed. Local tumor was excised and weighed. Tumor weight on the 35th day was significantly higher than on the 21st day. Data presented as the mean \pm standard deviation from six individual mice in each group. (A,B) Representative photo of local tumor on the 21th and 35th days. (C) Local tumor weight on the 35th days was significantly higher than the 21th days. Data presented as the mean \pm standard deviation. **, $P < 0.01$ 21 vs. 35 days. (D) Representative photo of local tumor on the 35th day between control and model group. CRC, colorectal cancer; MSOI, microsurgical orthotopic implantation.

Table 1 Local tumor take rate and metastatic rate in other organ[†]

Days	Local tumor take rate	Liver	Stomach	Abdominal wall	Retroperitoneal lymph node
21 d	6/6	5/6	0	0	0
35 d	6/6	6/6	3/6	6/6	6/6

[†], 6 mice on 21 and 35 d differently for observed.

dissemination foci, such as liver, stomach and abdominal wall, were analyzed by immunohistochemical staining. The results showed that local tumors invaded the normal cecum in both tangential and transverse directions to yield tumors. Liver tumor foci were also detected because there are so many sites for blood-borne metastases in human CRC. The results showed liver foci with tumor cells within and around blood vessels invading liver parenchyma. Stomach tissue and abdominal wall tissue assayed showed that tumor cells invade the mucous layer and placenta percreta (Figure 5).

Discussion

With cancer morbidity increasing rapidly over the last 10 years, mice models of many kinds of cancer, including

CRC, have been established. For the purpose of illuminating cancer progression and metastasis, cancer mice model research has turned from subcutaneous transplantation to *in situ* (12). A stable metastatic mouse model is essential to developing anti-cancer drugs and studying tumor progression mechanisms. This study established a stable hepatic metastasis of CRC mouse model with MSOI that can observe body conditions, tumor growth and metastasis at different time intervals. The method of sewing murine CRC tissue into the mucosa of the cecal wall of BALB/c mice generates models with dissemination patterns that closely replicate relevant metastatic sites observed in humans (13). However, the preliminary experiment results showed tumor tissue from CT-26 cell subcutaneous transplantation nude mice can produce

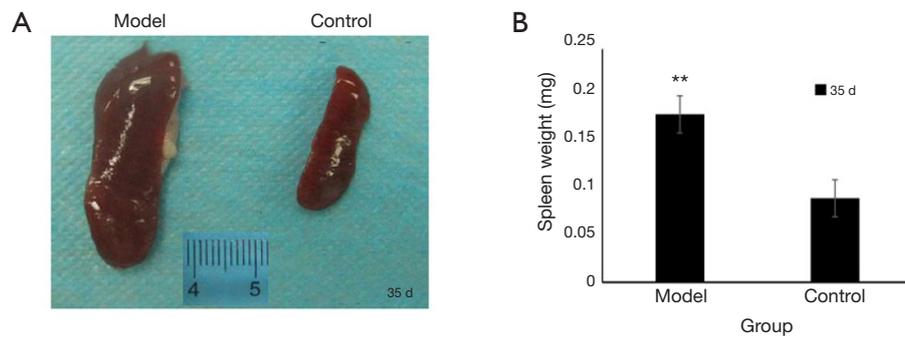


Figure 3 Spleen weight of orthotopic CRC mouse model on the 35th day. On the 35th day after mouse model was established by MSOI, mice were sacrificed. Spleen was excised and weighed. Spleen weight of model group was significantly increased over control group. Data presented as the mean \pm standard deviation from six individual mice in each group. **, $P < 0.01$ vs. controls (B). Representative photos of spleen (A). CRC, colorectal cancer; MSOI, microsurgical orthotopic implantation.

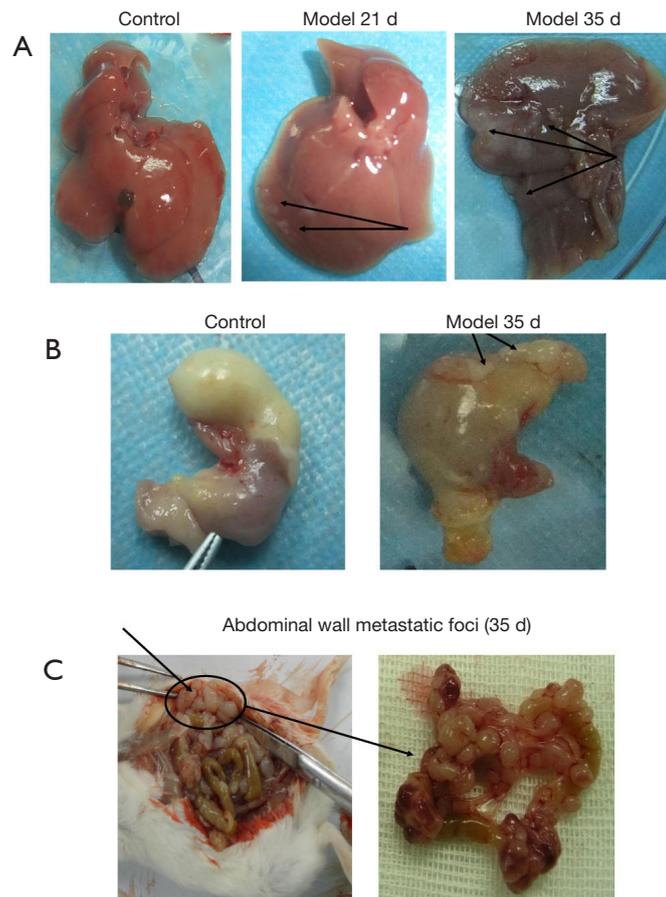


Figure 4 Metastatic tumor of orthotopic CRC mouse model. After mouse model was established by MSOI, on the 35th day, mice were sacrificed. Metastatic tumor burden was assessed. (A) Representative photos of liver metastasis; (B) representative photos of stomach; (C) representative photos of abdominal wall. Tumor nodules on different organ are indicated by arrows. CRC, colorectal cancer; MSOI, microsurgical orthotopic implantation.

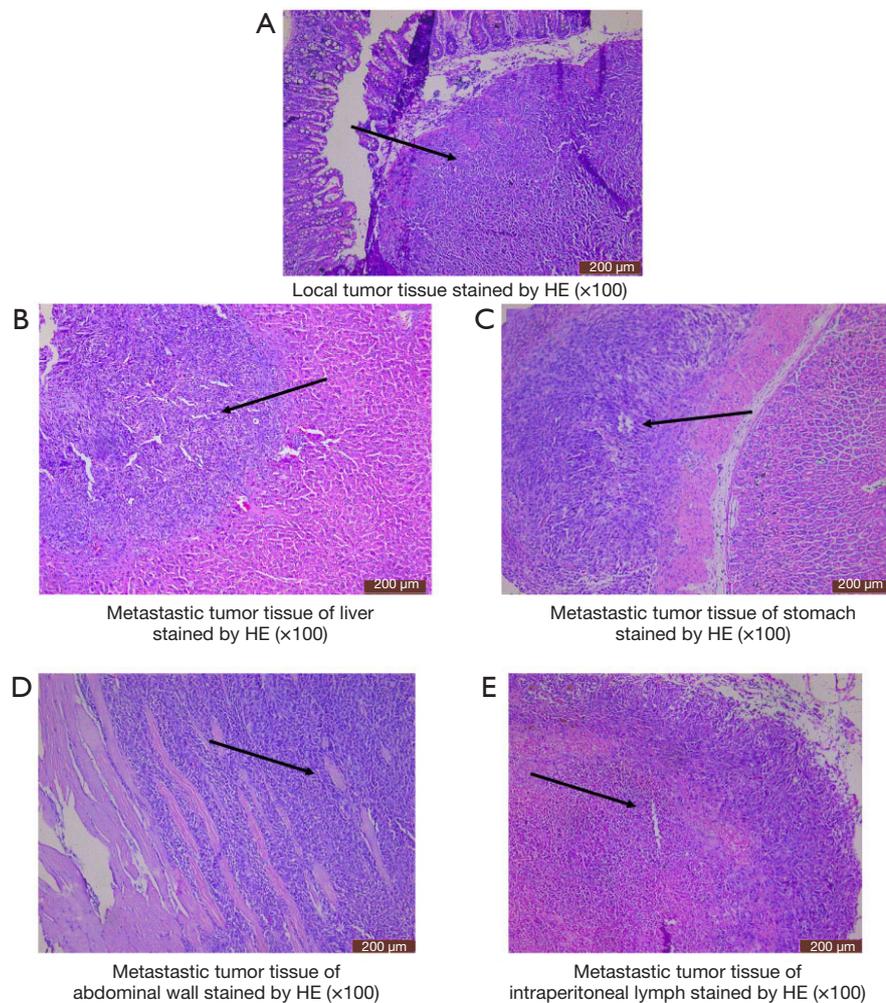


Figure 5 HE staining of tumor tissue for pathologic analysis. After mouse model was established by MSOI, on the 35th day, mice were sacrificed. Tumor tissue was sectioned, paraffin-embedded, and HE stained. Histopathological changes were observed under a light microscope. Tumor infiltration of different organ indicated by arrows. Images captured at 100× magnification (A,B,C,D,E). MSOI, microsurgical orthotopic implantation.

CRC along with metastatic nodes on different organ sites only within six generations. When CT-26 cell line was transplanted to nude mice in excess of seven generations, tumor tissue would not develop orthotopic colorectal tumor. Until now the reason is not clear but probably due to CT-26 cells growth and invasion ability reduction too low to produce orthotopic colorectal tumor with subcutaneous transplantation generations accumulation.

CT-26 cell local tumor growth showed relatively high take rates, showing 100% take on the 21st 35th days. Other researchers have reported local tumor take rates of human CRC cell lines (HCT-116, SW-620, and DLD-1)

in the 75% to 88% range with orthotopic microinjection transplantation. According to the results of HE staining, the local tumor pathological characteristics are very similar to those in human disease. For this reason, our orthotopic colorectal tumor mouse model of CRC *in situ* is a good animal model for new drug research.

Moreover, a key important site of colorectal tumor dissemination is liver and hepatic metastasis usually is a vital reason of patients death. Some papers have reported that human colorectal cell lines which express epidermal growth factor receptor can generate tumor foci in liver (14,15). Our results showed that on the 21st d and 35th d CT-26 tumor

tissue successfully developed hepatic metastasis by MSOI and metastatic rates were high than reported before (16-18). CT-26 mouse model of MSOI on the 21st d is adaptive for early liver metastasis because the metastatic nodes were relative small and no systemic metastases. Our team used CT-26 mouse model of MSOI on the 21st d to evaluate the effect of anti-liver metastasis of Pien Tze (11). And on the 35th d except liver metastasis, mouse models also showed ascitic fluid and metastasis nodes on stomach and abdominal wall. That characteristic mouse model can be used to detected advanced metastasis of colorectal tumor. However stomach metastases are rare and less reported. The feasible reason was that stomach adhered with peritoneal metastasis and will be studied further in the future.

In conclusion, the MSOI technique is a useful method to generate early- as well as advanced-stage liver metastasis of CRC with high take rates that can be achieved within a short time interval (one to two months). Therefore, this technique complements and expands the set of animal models currently available to CRC disease and treatment researchers.

Acknowledgments

Funding: This study was supported by Natural Science Foundation of Fujian Province (2017J01542, 2019J01727), The National Natural Science Foundation of China (81873166, 81774121).

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2020.03.61>). The authors have no conflicts of interest to declare.

Ethical Statement: We are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal treatments were performed strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

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Cite this article as: Cao Z, Zhang Z, Zheng L, Lin J, Peng J, Lin W. Establishment of a stable hepatic metastasis mouse model of murine colorectal cancer by microsurgical orthotopic implantation. *Transl Cancer Res* 2020;9(5):3249-3257. doi: 10.21037/tcr.2020.03.61

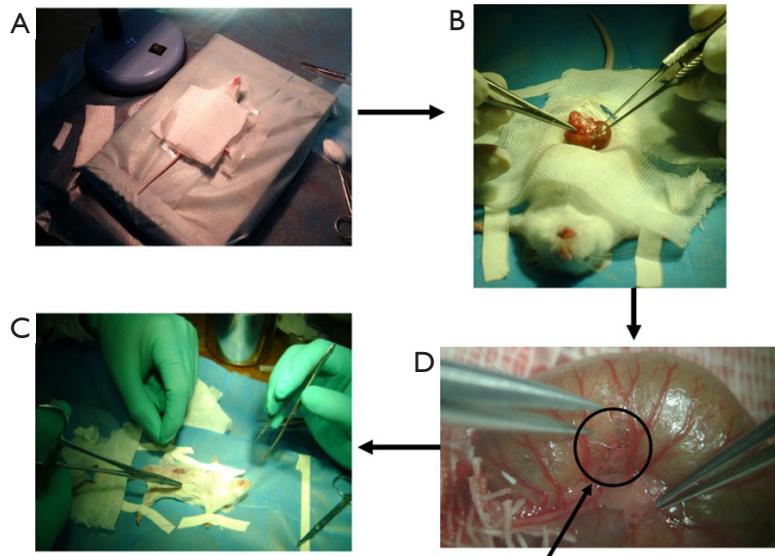


Figure S1 The procedure of MSOI. Mice were anesthetized by intraperitoneal injection with 1% pentobarbital sodium and secured on the operating table after hair was shaved (A). The cecum was elevated out and placed on sterile gauze alongside the incision (B). The operating table was moved under the microscope. A piece of tumor tissue derived from CT-26 was sewn in between mesenteric and muscular layers (C). The abdominal wall was closed in two layers with Dexon 5-0 (D).

Table S1 Alanine transaminase (ALT) and aspartate transaminase (AST) value of control and model group mice[†]

Days	Control group (U/L)	Model group (U/L)
21 d		
ALT	19.35±2.18	17.40±0.44*
AST	17.61±3.06	19.71±0.71*
35 d		
ALT	18.21±0.64	17.23±1.37*
AST	16.14±3.55	25.31±5.82*

[†], 6 mice every group for observed and data represent mean ± SD; *, P>0.05 control vs. model.