PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Orthotopic transplantation model of human gastrointestinal cancer and detection of micrometastases

Jun Hui Cui[1], Uwe Krueger[2], Doris Henne-Bruns[2], Bernd Kremer[2] and Holger Kalthoff[2]

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Abstract

AIM To establish a relevant animal model of human gastrointestinal cancer, which can be used for repetitive investigations, so as to improve our understanding and management of carcinogenesis and cancer metastasis.

METHODS Intact tissues of human colorectal and pancreatic cancers were transplanted in nude mice. The biological characteristics of the original and the corresponding transplanted tumors were investigated by HE staining, PAS staining and immunostaining. The metastases in the livers and lungs of nude mice were investigated by immunostaining with biotinylated mab KL-1 and by RT-PCR using CK20 specific primers.

RESULTS There were totally 9 of 16 surgical specimens growing in nude mice subcutaneously and/or orthotopically (4 of 6 colorectal and 5 of 10 pancreatic cancer). Tumor cell content of the specimens and freezing of tissue specimens are important factors influencing the growth of transplanted tumor. In the group of fresh tumor tissues with greater than 50% tumor cell content, the success rate of the transplantation was 100% (3 cases of pancreatic cancer and 3 cases of colorectal cancer). The orthotopically transplanted tumors resemble the original tumor morphologically and biologically, including TAA expression such as CEA by immunohistochemistry, and CEA level in the serum of mice. Ki-67 labeling index and the expression of TAA especially K-ras, 17-1A and RA-96, are associated with the potential of tumor growth in nude mice. Micrometastases in the lungs and livers of tumor bearing mice can be detected by immunostaining with biotinylated mab KL-1 and CK20-specific RT-PCR.

CONCLUSION An orthotopic transplantation model for human colon and pancreatic cancer in nude mice has been set up. We have also established sensitive detection methods with CK-immunohistochemistry and CK20-RT-PCR to study xenotransplanted human cancer and its metastatic cancer cells in the liver and lung of nude mice. This study may be helpful in understanding the mechanism of cancer metastasis and in developing new diagnostic methods and therapeutic strategies for metastases including micrometastases.

[1]Department of General Surgery, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
[2]Department of General Surgery, Christian Albrechts University, Kiel, Germany
Dr. Jun Hui Cui, graduated from Zhejiang Medical University in 1984, earned master degree in 1990, studied in the Surgical Department of Kiel University and worked in the Lab of Molecular Oncology of Kiel University from 1994-1997, and achieved M.D. from Kiel University, Germany, now associate professor of surgery, specialized in colorectal oncology. Adviser of graduated student for master degree, having 20 publications published in key Chinese or English journals.
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Correspondence to: Dr. Jun Hui Cui, Department of Colorectal Surgery, the First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China
Tel. 0086-571-7236882, Fax. 0086-571-7072577
Email. Cjhui@mail.zj.hz.cn
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INTRODUCTION

Despite significant improvement in surgical techniques and apparently curative resection, recurrence and metastasis still occur and is the leading cause of solid malignant tumors[1-7]. Current standard diagnostic techniques are not able to detect early dissemination of cancer cells (micrometastases)[8-10], and conventional classification of tumor stages cannot account for the presence or absence of distant micrometastasis in the patients with small primary tumors. Thus, one of the most critical prognostic determinants for the subsequent clinical course is neglected in many patients[9,11-16]. Specimens from individual patients are often difficult to obtain for detailed analyses, but it will be much easier to investigate materials in animal models, for studying the various aspects of tumorigenesis, especially metastasis because any part of the tissues from the model can be taken for

detailed investigation.

Most models based on athymic nude mice have been playing an important role in evaluating many anti-cancer drugs. However, human tumors were transplanted subcutaneously and not at an orthotopically relevant organ site. The major problem of this model is that the transplanted tumors are located in an environment quite different from where most human tumors locate. Most subcutaneously transplanted tumors are surrounded by a pseudocapsule, and have little chance to invade and disseminate to the surrounding tissues and rarely metastasize, even when highly aggressive tumors have served as the source of the xenograft[17]. However, human tumor cells implanted orthotopically in the corresponding organs of nude mice can increase the metastatic capability of human tumor cells in nude mice[17-23]. As a relevant model of human gastrointestinal cancer, orthotopically transplanted tumors in nude mice can improve our knowledge about invasive growth and metastasis. Such tumor model would be available for repeated investigation of experimental diagnosis and treatment of early metastasis. It may help design more effective therapeutic strategies[6,19,22-24].

MATERIALS AND METHODS

Specimens and animals

Tumor specimens for transplantation were taken from primary lesions in pancreas, colon and rectum. Surgical tumor specimens which were directly taken from patients were aseptically removed during surgery and immediately transported and processed. Each specimen was separated into three parts: one part for transplantation of fresh specimen, one part for subsequent transplantation of DMSO-frozen specimen (mostly in 1d to 3d), and another part was stored at-80℃ for morphological, immunohistochemistry analyses and for RTPCR. Eight to twelve week old male or female athymic NMRI nude mice, which were obtained from the University Clinic Eppendorf, Hamburg, Germany, were kept in laminar-flow cabinets.

Surgical procedures of tumor transplantation

Each cancerous tissue (fresh or frozen tissue) was divided into small pieces about 2 mm in diameter. Mice were anesthetized with a mixture of 12 $g \cdot L^{-1}$ ketamin Curamed Phama GmbH) and 1.6 $g \cdot L^{-1}$ xylazin solution (Parke-Davis GmbH), The colon or pancreas was then carefully exposed and a tumor piece was then attached on the serosal surface of the colon or the wall of the pancreas, with 6-0 absorbable transmural suture. The colon or pancreas was then returned to the peritoneal cavity and the abdominal wall and the skin was closed with 6-0 absorbable suture. Immediately thereafter, another piece of the tissue was transplanted subcutaneously into the left frank of the respective mouse. For the purpose of convenience, the DMSO-frozen tissues were also transplanted into another 2-3 mice. Tumor pieces from each patient were transplanted into 2-3 mice in general.

Immunohistochemistry

A modified immunoperoxidase procedure, which was introduced in 1981 by Hsu *et al*, was used as follows. Tumor sections were air dried, fixed with acetone for 10 minutes prior to staining, transferred into PBS, blocked for 20 minutes with diluted normal horse serum, incubated for 30 minutes with primary antibody diluted in 10 $g \cdot L^{-1}$ BSA/PBS, washed for 5 minutes in PBS (if quenching of endogenous peroxidase was required, the sections were incubated for 30 minutes in methanol with 3 $mL \cdot L^{-1}$ H_2O_2), incubated for 30 min with a diluted biotinylated secondary antibody solution (biotinylated horse anti-mouse IgG 1:200 in 15 $mL \cdot L^{-1}$ horse serum/PBS). If the primary antibody was C-T84.66, biotinylated goat anti-human IgA+IgG+IgM (Jackson Inco. 1:1000) was used, washed for 5 min in PBS, incubated for 30 min with "VECTORSTAIN ELITE ABC Reagent", washed for 5 min in PBS, incubated for about 5 min in DAB solution, rinsed in tap water, counterstained and cleared, airdried and mounted. Positive and negative controls were included as mentioned above.

Monoclonal antibodies

mAb KL-1 and Biotinilated KL-1 anti-cytokeratin (IgG 1, kappa, 4 $mg \cdot L^{-1}$, Immunotech, Hamburg, Germany); CIP83 (5 $mg \cdot L^{-1}$, Kalthoff, Kiel, Germany) and chimeric-T84.66 (1:100, Neumaier, Hamburg, Germany) anti-CEA (IgG1, kappa); and MIB1 (2.5 $mg \cdot L^{-1}$) and Biotinilated MIB1(10 $mg \cdot L^{-1}$) anti-ki-67 (IgG 1, Dianova, Hamburg, Germany) were used both for investigating original and transplanted tumors. mAb CA-199, Ra-96 and 17. 1A (all 5 $mg \cdot L^{-1}$, Kalthoff, kiel, Germany) were used for investigating human original tumor only, Biotinilated KL-1 was also used for detecting micrometastatic cells in the livers and lungs of mice.

Detection of CEA level in mice serum

Serum from nude mice were measured using a Microparticle Enzyme Immunoassay (MEIA) for the quantitative measurement of CEA with the "IMx" immunoassay analyzer (Abbott Laboratories).

Micrometastases detected by CK20-specific RT-PCR

Livers and lungs of nude mice were cut into 10-20 μm slides with cryosection machine, then RNA was extracted using modified single-step RNA isolation

method with TRIzol (Gibco BRL, Eggenstein, Germany). The integrity of RNA was checked by gel-electrophoresis. RNA extracted from a pancreatic carcinoma cell line A818.4 was used as positive control, GAPDH (glycerdehyde-3-phosphate dehydrogenase) -RT-PCR was used to monitor cDNA synthesis. The detailed protocol of ck20-specific RT-PCR was described previously[25,26].

RESULTS

Mice were killed when transplanted tumors reached 1 cm or larger. There were 4 (67%) out of 6 colon cancer, 5 (50%) of 10 pancreatic cancer, totally 9 (56%) of 16 surgical specimens growing in nude mice sc and/or orthotopically. Tumor growth was observed in 0% of frozen tissues and in 63% of fresh tissues with paired samples of original tumors. When retransplanted with xeno-transplanted tumors, 39% of transplants of frozen tissues and 100% of fresh tissues grew in the mice (Table 1). In the group of fresh tumor samples with a tumor cell content ⩾50%, the success rate was 100% (6/6). While in the group with a cell content of <50%, the success rate was 40% (2/5), (Table 2). The expression of Ki-67, K-ras, 17.1A and Ra-96 antigens in human colorectal and pancreatic cancer with tumor growth in nude mice was higher than in those without tumor growth (Figures 1, 2). Almost the same morphology and CEA expressions were observed between original human tumors and corresponding xenotransplanted tumors of colon and pancreatic cancer (Figures 3-8). The serum CEA levels of mice are closely associated with the existence of tumors in nude mice (Table 3). Micrometastases in lungs and livers of tumor-bearing mice: 16 mice transplanted with colorectal, and pancreatic carcinomas from 7 cases of original tumors (5 of the 7 cases had corresponding orthotopic tumors) were investigated for liver and lung micrometastases. In the 16 mice, which were sacrificed around 13-14 weeks after transplantation, macroscopically invisible metastases were found in the lungs of 3 mice by KL-1 immunostaining and CK20-RT-PCR (2 colorectal and 1 pancreatic cancer). Additionally 2 liver metastases from colorectal cancer were detected by CK-20 specific RT-PCR only (Figures 9-11).

Table 1 Tumor growth of paired fresh (F) and DMSO-frozen (D) tissue: transplanted with original tumors and xenotransplanted tumors[a]

Type	TG / total (*n*)		Success rate (%)	
	F	D	F	D
Colon	3 / 3 (12 / 12)	0 / 3 (6 / 10)[b]	100 (100)	0 (60)
Pancreas	2 / 5 (8 / 8)	0 / 5 (1 / 8)	40 (100)	0 (13)
Total	5 / 8 (20 / 20)	0 / 8 (7 / 18)	63 (100)	0 (39)

[a]Figures in ( ) is the results of transplantation with xenotransplanted tumors; b1 mouse died after transplantation.

Table 2 Influence of tumor cell content on tumor growth of fresh surgical specimens

Tumor	TG / total (*n*)		Success rate	
	⩾50%	<50%	⩾50%	<50%
Colon	3 / 3	1 / 1	100	100
Pancreas	3 / 3	1 / 4	100	25
Total	6 / 6	2 / 5	100	40

Table 3 Serum CEA levels of mice with or without tumor

	With tumor($\bar{x} \pm s$, mg·L^{-1})	Without tumor($\bar{x} \pm s$, mg·L^{-1})	*P* value
Colon	19.33±28.77 (*n* = 17)	0.1 ± 0.06 (*n* = 7)	<0.005
Pancreas	2.85±1.89 (*n* = 11)	0.29 ± 0.2 (*n* = 7)	<0.005

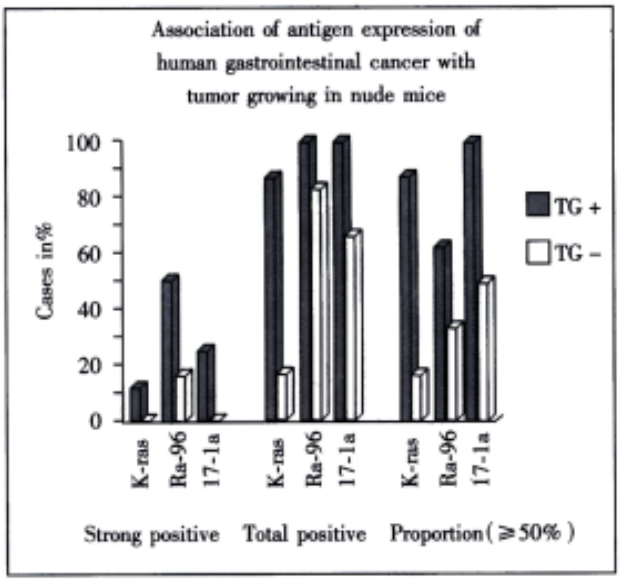


Figure 1 Ki-ras, Ra-96 and 17.1a antigen expression of original tumors.
Strong positive: with the positive intensity from "++" to "+++"
Total positive: with the positive intensity from "+" to "+++"
Proportion (⩾50%): ⩾50% of tumor cells in the section with positive staining
TG + (*n* = 8): = with tumor growth
TG - (*n* = 6): = without tumor growth

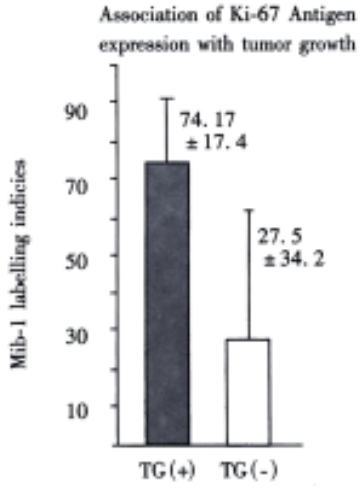
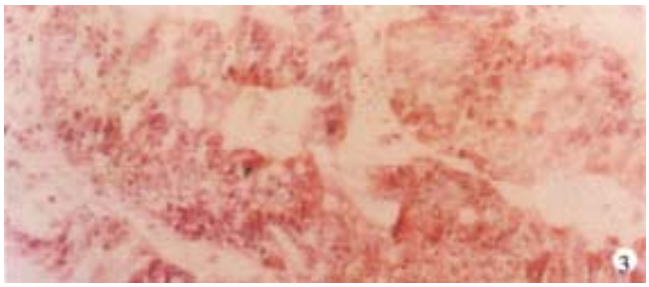
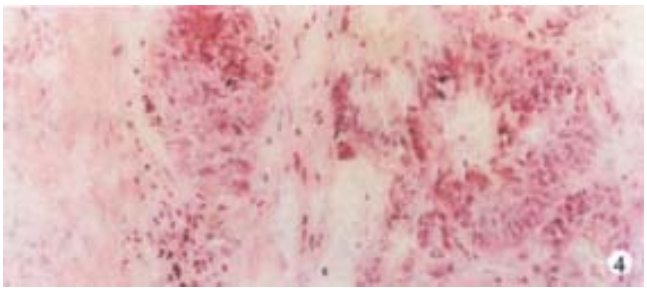
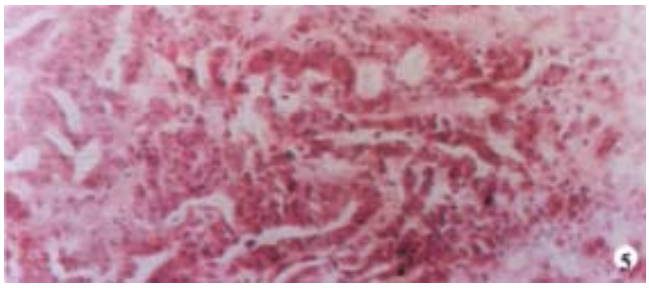
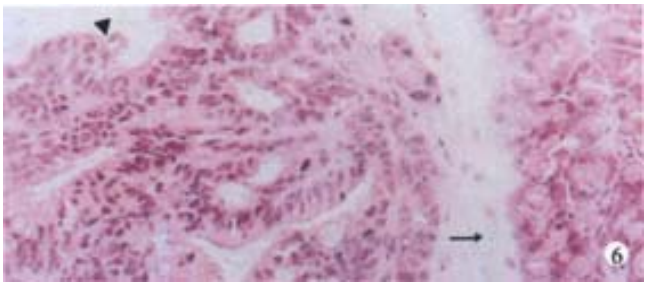


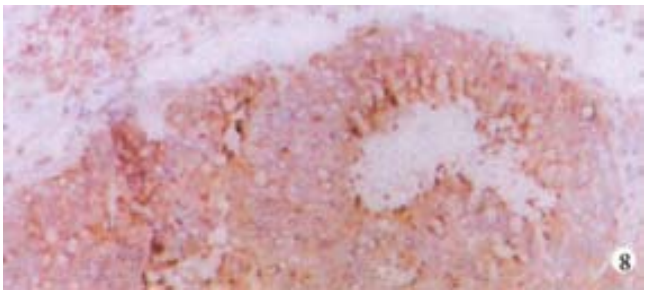
Figure 2 Ki-67 antigen expression of original tumors.
TG (+): 74.2 ± 17.4 (*n* = 9)
TG (-): 27.5 ± 34.2 (*n* = 7)
(*P*<0.005)

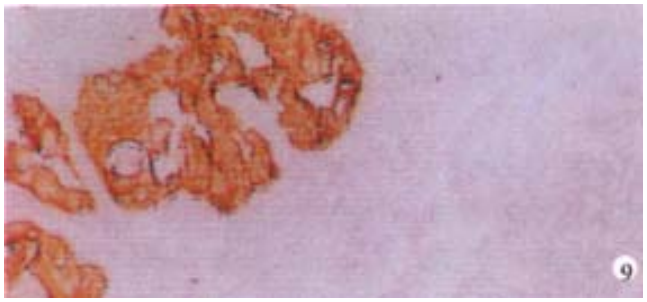












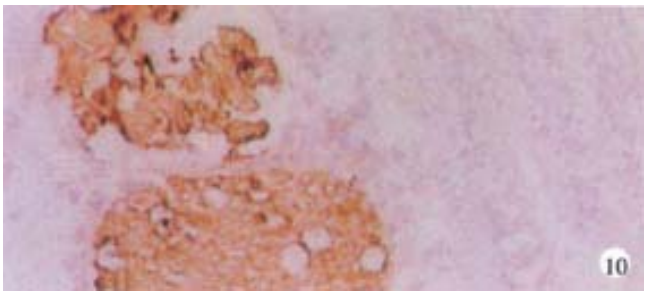


Figure 3 Human colon cancer. HE×20
Figure 4 transplanted orthotopic cancer. HE×20
Figure 5 Human pancreatic cancer. HE×20
Figure 6 Transplanted orthotopic pancreatic cancer. HE×20
Figure 7 CEA expression of human colon cancer, with immunostaining. ×20
Figure 8 CEA expression of transplanted orthotopic colon cancer, with immunostaining. ×20
Figure 9 Transplanted orthotopic colon cancer, with immunostaining staining. ×20
Figure 10 Metastatic colon cancer in the lung of nude mouse, with immunostaining. ×20

Figure 11 Specimens of 2 μg total RNA were subjected to CK-20 specific RT-PCR (485 bp product).
M. DNA molecular marker; 1:sc tumor (case A); 2:Orthotopic tumor (case A); 3:Liver (case A); 4:Lung (case A); 5:sc tumor (case B); 6:Orthotopic tumor (case B); 7:Liver (case B); 8:Lung (case B); 9:H2O; 10:A818.4 cell line (50 $\mu g \cdot L^{-1}$); 11:A818.4 cell line (50 $\mu g \cdot L^{-1}$) without reverse transcriptase.

DISCUSSION

Developing a relevant animal model of xenotransplantation of human tumors in nude mice could improve our understanding of the biological properties of human gastrointestinal cancer and lead to the development of new effective therapeutic concepts. Human tumor xenografts transplanted SC in athymic nude mice rarely give rise to metastases [24]. However, human tumor cells implanted orthotopically in the corresponding organs of nude mice can increase the metastatic capability of human tumor cells in nude mice[18,24]. Data have shown that desegregated tumor cell suspensions may not always express their fully malignant biological behavior[17,27]. Cell suspensions may have the possibility of seeding tumor cells "artificial" metastases in the surrounding tissues (i.e. peritoneal cavity) during the process of inoculation. Moreover, inoculation of desegregated tumor cell suspensions does not reflect the situation when metastatic cells spread from intact tissue growing in the human body.

Therefore, we have established an orthotopic transplantation tumor model for human tumors in nude mice with intact tissues of pancreatic, and colorectal cancer. The results showed similar morphology and biological behavior before and after transplantation in this model. Orthotopical transplanted tumors present many of the clinical manifestations of the biological behavior of gastrointestinal cancer in humans, including invasion, metastasis, and antigen expressions of CEA, CK, Ki-67, etc. CEA is a most commonly used tumor marker for gastrointestinal cancers. Recent studies showed that CEAmRNA RT-PCR or CEA immunostaining is frequently used in detection of micrometastases of gastrointestinal tract cancers [1,12,28,29]. CEA levels in serum play an important role in monitoring patients with gastrointestinal cancer [30-34]. We found that serum CEA level is obviously elevated in tumor bearing mice, suggesting that serum CEA level is a very useful marker in this model. KL-1 reacts with cytokeratins (CK), components of intracytoplasmic network of intermediate filaments (Ifs)[4,8,35], specifically reacts with epithelial tumors. Anti-CK mABs using immunocytochemistry or CK RT-PCR have been widely used for the detection of micrometastases in lymph nodes, venous blood and bone marrow[1,4,12,28,36-42]. It is satisfactory using KL-1 monoclonal antibody to detect micrometastasis in the lungs and livers of the mice in this study. Ki-67, aproliferation-associated antigen [43-46], is expressed in all phases of the cell cycle (G1, S, G2, and M) except for G0[47]. It is thought to be a useful predictor of aggressive tumor behavior and an indicator of patient survival[43]. Mib-1 is raised against recombinant parts of the Ki-67 antigen[48-52]. Our results showed that the original tumors with higher Mib-1 (Ki-67) labeling index had an increased tendency to grow in the nude mice.

Meanwhile, we established the sensitive method to detect disseminated tumor cells (micrometastasis). Sixteen tumor-bearing mice (with 5 orthotopic transplanted tumors) were used for investigating the metastasis of livers and lungs. There was no obvious metastases with naked eye and HE staining but 3 lung micrometastases with KL-1 immunostaining and CK20 RT-PCR, additionally 2 liver metastases with CK20 RT-PCR, suggesting that RT-PCR may be more sensitive than immunostaining. Many factors influence tumor growth in nude mice. On the one hand, tumor tissues are composed of cells with different biological characteristics[7]. On the other hand, different mice may have various reactions to transplantation.

Our data includes the frozen tumor tissue implantation, because the procedure is more flexible and convenient. Unfortunately, freezing of tissue specimens obviously reduced the success rate. However, for tumors with a good cellularity, freezing is a possible procedure. When xenograft tumor tissues were retransplanted (xenografts were taken from mice and frozen, then thawed and transplanted again), the tumor growth rate of pancreatic and colorectal frozen tumor tissues, was nearly 40% and only the colorectal tumors was 60%. This is likely due to the fact that xenograft tumor tissues contain more tumor cells and less damaged cells due to shorter storage time and better preservation. Tumor cell content is an important factor in our study. In the specimens with a tumor cell content greater than 50% (tumor cells vs stroma over 1:1), the success rate of fresh tumor tissues impliantation reached 100% (Table 3).

In this study, we found that TAAs, including C1P83, RA-96, 17.1A, K-ras and Ki-67 expression, were important indicators of tumor growing potential in nude mice.

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