

• LIVER CANCER •

Nude mice model of human hepatocellular carcinoma via orthotopic implantation of histologically intact tissue

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Abstract

AIM: To establish a nude mice model of human hepatocellular carcinoma (HCC) via orthotopic implantation of histologically intact tissue, in order to study biologic features of HCC *in vivo* and to direct clinical treatment respectively.

METHODS: Histologically intact fresh specimens of HCC were orthotopically implanted in nude mice (BALB/c, nu/nu). Survival rate and growth curve were investigated with B-ultrasound. Morphological characteristics of pathology and spontaneous metastatic rates were detected with microscopy. Expression of multidrug resistance genes studied with immunohistochemical method and RT-PCR, and other biologic features of implanted tumor were observed and compared with human HCC specimens.

RESULTS: Out of the specimens from two patients with HCC, only one specimen survived in nude mice. The orthotopic implantation tumor survival rate, spontaneous intrahepatic metastatic rate, pulmonary metastatic rate and bone metastases rate were 100%, 75.0%, 37.5% and 37.5% respectively in the first passage. AFP was kept on secreting and increasing with the size of the tumor. The morphological characteristics and biologic features were similar to the donor's, the protein and mRNA of MDR1 and LRP were expressed in tumors of the model and the donor, and there was no significant difference between them ($P > 0.05$).

CONCLUSION: The model of nude mice with orthotopic implantation of histologically intact HCC tissue is an ideal model to study biologic features of HCC *in vivo* and to direct clinical treatment.

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INTRODUCTION

Liver cancer is one of the most common carcinomas, the highest

age-standardised mortality rate in China, and accounts for 53% of all liver cancer deaths worldwide^[1,2]. Surgical resection has been accepted as the best treatment for hepatocellular carcinoma (HCC). However, recurrence and metastases and non-sensitivity to chemotherapy remain the major obstacles for further prolonging survival after resection^[3]. Study of HCC, therefore, has become an important issue. But the experiments can not be done on patients with HCC. So human HCC model in nude mice is needed for the study of HCC and its mechanism, chemotherapy, multidrug resistance (MDR), *etc.*

Shimosato *et al.*^[4,5] established a series of human tumors in nude mice including the human HCC nude mice model for study of alpha-fetoprotein (AFP) in relation to tumor growth in 1976. Liu *et al.*^[6] established a nude mice xenograft model from human HCC in 1995. Leveille-Webster *et al.*^[7] established an intrahepatic xenograft of human HCC in severe combined immunodeficiency mice for the study of multidrug resistance in 1996. Sun *et al.*^[8,9] reported a metastatic human HCC model in nude mice with 100% of spontaneous metastases to lung, lymph node and liver. Peng *et al.*^[10] established a human HCC model in nude mice using orthotopic implantation and observed malignant behavior. Tao *et al.*^[11] established a human HCC in nude mice model using SMMC-LTNM tumor transplanted into abdominal cavity and liver, the lung metastatic rate was 59%. Genda *et al.*^[12] reported the construction of metastatic models using orthotopic implantation of human HCC cell lines into livers of SCID mice, two of the 5 cell lines injected showed vascular tumor thrombi and intrahepatic metastases. Zheng *et al.*^[13] established an orthotopic implantation tumor model from the subcutaneous model of human HCC in nude mice, the spontaneous metastatic rate was 57.8%. Shi *et al.*^[14] established a human HCC model in nude mice with a high metastatic rate in lymph node.

Metastatic models constructed in nude mice by orthotopic implantation of histologically intact patient specimens have been used in Hoffman's group, and several such models including cancers of the lung, pancreas and ovary, have been reported^[15-17]. However, patient-like human HCC model in nude mice with metastatic behaviors was not found. After the study on HCC cell lines and subcutaneous HCC model in nude mice, we established a patient-like human HCC model with spontaneous metastases in liver, lung and bones.

MATERIALS AND METHODS

Mice

There to four weeks BALB/c, nu/nu, nude mice (Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan), weighting 10.1-16.0 g, were used in this study.

Surgical specimens of human HCC

Fresh surgical specimens were obtained from 2 patients with HCC who underwent surgery at the Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The specimens were rinsed and preserved in saline at 4-8 °C and sent to the Experimental Animal Center as soon as possible. After necrotic tissue and non-

cancerous tissue of the specimens were removed, the remaining cancerous tissues were cut into small pieces of about 1 mm³ in size.

Implantation procedure

Tumor pieces were implanted into the livers of nude mice. A left subcostal incision was made under anaesthesia with 200 g/L urethane. The left lobe of the liver was exposed and the liver capsule was mechanically injured with needle. Then one or two pieces of tumor tissue were filled into the liver tissue with forceps which could be seen as a white spot and abdominal wall was finally closed. Mice were kept in laminar-flow cabinets under specific-pathogen-free conditions.

Evaluation of growth and metastases

Nude mice were weighted and the size of tumors was weekly measured with B-ultrasound from 2 to 9 wk after orthotopic implantation. Tumor size (mm³) was evaluated by measurement of two diameters with B-ultrasound and using the formula $1/2 LW^2$, where L is the longest diameter and W is the shortest diameter. Bone metastases was detected with SPECT 8 wk after implantation and then other organ metastases was detected with autopsy and microscopy when the mice were killed. The liver, lymph node, lungs, and bone suspected of metastases were collected and processed for routine gross and microscopic examination. Metastases was diagnosed if at least one microscopic metastatic lesion was found in any resected organ.

Serum detection

AFP in serum from tail vein was detected every two weeks, and hepatitis virus-B surface antigen (HBsAg), antibody to hepatitis virus-B surface (HBsAb), hepatitis virus-B e antigen (HBeAg), antibody to hepatitis virus-B e (HBeAb) and antibody to hepatitis virus-B core (HBcAb) of serum from heart blood were detected after mice were killed (ELISA assay).

Immunohistochemical studies

Expression of MDR1 and LRP was studied by using mouse anti-human MDR1 C219 and LRP monoclonal antibodies (neomarkers) respectively. Tumor tissues from patients with HCC and mice carrying human HCC were fixed in formalin and embedded in paraffin. Sections of 5 µm were assayed for MDR1 and LRP expression by immunoperoxidase staining with the SP method (SP kit, Zhongshan Biocompany, China).

Table 1 Primers for MDR1 and LRP

Gene	Size (bp)	Quantification method	Sequence (5'-3')
B-actin	530	Forward primer	GTGCGTGACATTAAGGAG
		Reverse primer	CTAAGTCATAGTCCGCCT
MDR1	174	Forward primer	CATTGGTGTGGTGAGTCAGG
		Reverse primer	CTCTCTCTCCAACCAGGGTG
LRP	237	Forward primer	TAAGGGCTTCCAGCACCAAC
		Reverse primer	GGAGTTCTCGTTCTCGTCC

Detection of MDR1 and LRP expression by RT-PCR

Total RNA was extracted from tumors of nude mice and patients using Trizol (Promega) according to the manufacturer's instructions. Reverse transcription (RT) was performed with random primers using a complementary DNA (cDNA) synthesis kit (promega). Following RT-reaction reagents were added: 2 µL MgCl₂ (50 mmol/L), 2 µL RT buffer (Tris-HCL pH 8.3), 2 µL deoxynucleotide mixture (100 mmol/L), 0.5 µL RNase (20 units), 1 µL M-MLV reverse transcriptase, 1 µL of random primers (500 µg/mL) and 5 µg substrate RNA. The final volume of RT-reaction (25 µL) was completed with RNase free water. First strand cDNA synthesis was carried out at 37 °C for 60 min in a

DNA thermal cycler. Afterwards, the tubes were incubated at 95 °C for 5 min to terminate the reaction. Then each tube was kept at -20 °C until PCR was performed. Primers of the target gene MDR1, LRP and endogenous reference GAPDH were designed using the Primer Express software (Applied Biosystems) (Table 1). The final volume of PCR reaction (50 µL) was completed including an initial phase at 94 °C for 4 min, and followed by 30 cycles at 94 °C for 1 min, and at 56 °C for 45 s, at 72 °C for 45 s, and finally at 72 °C for 10 min.

Statistical analysis

Data were presented as mean±SD and analyzed with SPSS11.0. $P < 0.05$ was considered statistically significant.

RESULTS

Establishment of patient-like metastatic HCC model

Human HCC model of orthotopic implantation was successfully established in nude mice with histologically intact tissues. Out of the 2 specimens from 2 patients with HCC, only one specimen from a 39-year-old man with HCC was at Edmonson grade II-III, whose serum AFP (1 746 ng/mL), HBsAg, HBeAg, HBcAb were all positive, and he had extensive intrahepatic metastases but no hilus hepatis lymph node metastases during operation. Bone metastases were found in the patient one month after operation. Two weeks after implantation, all the livers of 8 nude mice gave rise to tumors and the size of tumors was observed every week with B-ultrasound or by autopsy after they were killed. (Figures 1-3).



Figure 1 Ultrasonic image of implanted tumors in nude mice liver.



Figure 2 Implanted tumors in nude mice liver by autopsy.

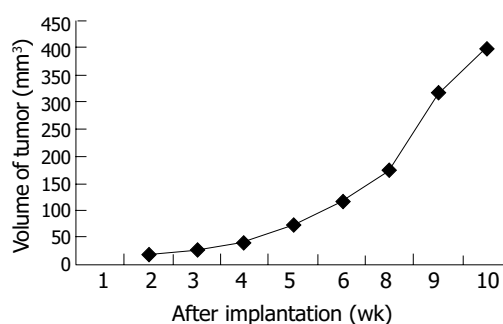


Figure 3 Relation between volume of implanted tumor and time after implantation.



Figure 4 Implanted tumor in nude mice liver metastases to lungs. The first passage just formed a few metastatic lesions A; The 4th passage formed multiple metastatic lesions B; and metastatic lesions in lungs were found under microscope C.

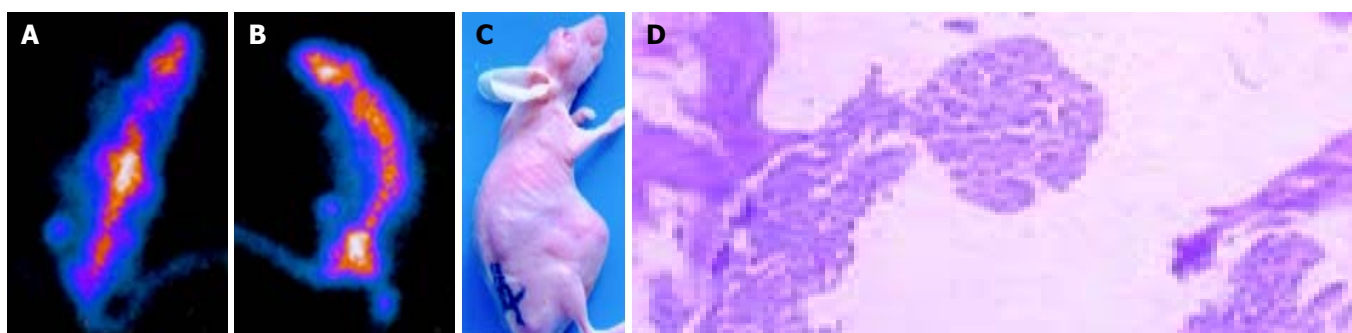


Figure 5 Implanted tumor in nude mice liver spontaneously metastasized to bone. The first passage metastasized to vertebra thoracica just as the donor A, and the 4th passage showed multiple bone metastases B, C. Metastatic lesions were shown in bones under microscope D.

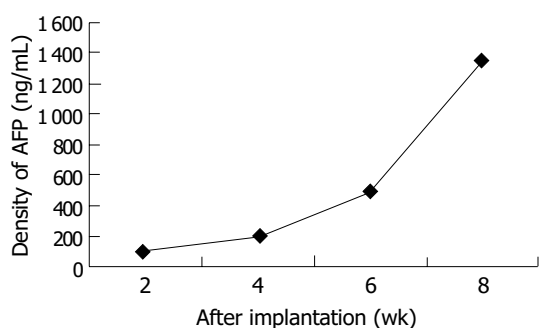


Figure 6 Relation between density of AFP in serum and time of tumor after implantation.

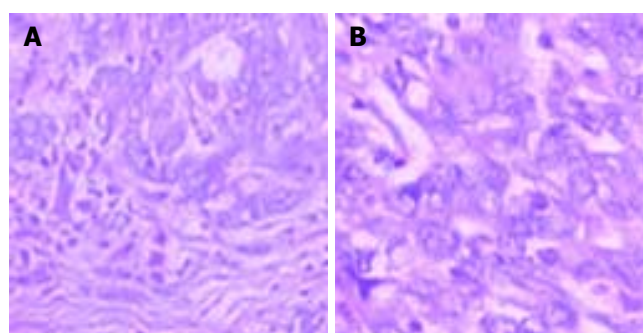


Figure 7 Edmonson grade II-III HCC specimen from patients A and in tumor tissue from nude mice model B.

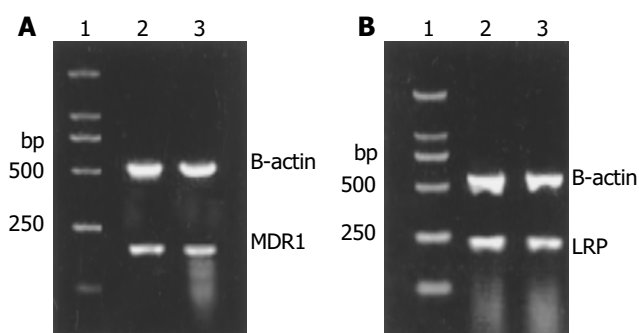


Figure 8 MDR1 and LRP expressed in tumors of the donor (2) and the nude mice model (3), and the expression had no significant difference between both. A: MDR1 expressed in tumors. B: LRP expressed in tumors.

The tumor behavior of this model was the same as the tumor in the patient who donated the specimen. Nine weeks after

implantation, the 8 nude mice with normal weight were killed by drawing blood from heart. According to autopsy and microscopic examination, implanted tumor survived in all nude mice (8/8), intrahepatic invasion was seen in all nude mice (8/8), intrahepatic metastases was seen 75% (6/8) (Figure 2), lung metastases was seen in 3 nude mice (35.5%) (Figure 4), the wound seeding was seen in all mice (8/8), and bone metastases occurred in 35.5% (3/8) (Figure 5), but lymph node metastases was not found.

Findings in serum

Serum AFP was detected from tail blood of nude mice (Figure 6), but all markers of hepatitis were negative.

Histological findings

Histological characteristics of the model tumor were similar to those of the donor's tumor (Figure 7).

Immunohistochemistry and RT-PCR results (Figure 8, Table 2)

Table 2 Expression of MDR1 and LRP in HCC from donor and nude mice

	Immunohistochemistry (%)		mRNA of RT-PCR (%)	
	MDR1	LRP	Mdr1	LRP
Donor	31.26	27.18	61.47	56.71
Nude mice	29.20±2.81	25.44±2.43	65.42±13.59	58.63±9.37
<i>t</i>	2.077	2.030	0.823	2.288
<i>P</i>	0.076	0.080	0.438	0.056

DISCUSSION

Subcutaneous tumor implantation has been used as a standard method to establish animal models of human cancer^[18,19]. Although such models help to understand the nature of human cancers and their therapeutic approaches, but many problems still remain unsolved. One major problem is that the tumor which was derived from a patient and implanted subcutaneous into an immunodeficient animal no longer behaves as it did in the patient. Although the tumor could sometimes grow subcutaneously, it was encapsulated and usually failed to metastasize either regionally or at distant site^[19].

Recently, a new strategy of "orthotopic implantation" has been used to develop rodent models of metastatic human cancer^[19,20]. In the first generation of these models, cell lines or disaggregated cells were injected into the organ of mice that corresponded to the organ from which human tumor was derived. This method allowed metastases to occur at least in certain cases^[19,21-23]. The cell line and disaggregated cells used for orthotopic implantation were obtained by disrupting the original structure of human tumor tissue, which might lead to a change in nature and biological behavior and could be the basis of the greatly reduced metastases rate^[24]. Holfman^[25] developed an orthotopic implantation model utilizing intact tissues obtained directly from surgery. This approach has yielded a high survive rate and frequent metastases in cancers of the colon^[26], bladder^[24], lung^[27], pancreas^[28], prostate^[29] and stomach^[30]. These models of human cancer in nude mice could show various manifestations similar to tumor behavior in patients.

However, it remains difficult to obtain satisfactory models for human cancers in nude mice, particularly with regard to spontaneous metastases and reduction of the latent period when cell suspension or histologically intact tissues were used for orthotopic implantation, which could not really reflect the nature of HCC of patients and preclude *in vivo* effective study of important events including spontaneous metastases and multidrug resistance. Therefore, our approach includes the selection of highly invasive and intrahepatic metastases of human HCC samples and orthotopic transplantation of histologically intact tissues into 3-4 wk old nude mice. In the first generation, the result demonstrated that the specimen from the 39-year-old patient with HCC gave rise to growth of tumor at the implantation site in all the 8 nude mice 2 wk after implantation. In the course of the study, lung and bone metastases were found in the patient donating specimens 1 mo after operation. So we began to pay attention to nude mice in terms of metastases. Fortunately, metastases occurred in all the mice 7-8 wk after implantation, 3 (3/8) had lung metastases, and 3 (3/8) had spontaneous bone metastases, and all had wound seeding and intrahepatic invasion. Lymph node metastases, however, was not found. Spontaneous metastases of human cancer xenografts in nude mice was rare^[9,11]. We believe the reasons of cancer growth and metastases in nude mice were that the specimen selected was highly metastatic and that the nude mice were younger (3-4 wk) than those used in other studies^[13,31]. But metastases was not found in all the mice. We believe the reasons might be that the time after implantation was not so

long that the metastases could not occur in each one, and that it was related to the methods detecting the metastases. So in the 2-4 passages, the nude mice carrying implantation tumor were not killed until they developed signs of distress so as to give full time to metastases and to observe the nature of the tumor. As a result, intrahepatic, lung and bone metastases and wound seeding and multiple metastatic sites in those organs were surprisingly observed in all nude mice over a period ranging 5-16 wk after implantation. Besides selection of the highly invasive and metastatic samples and age of the nude mice, we think there were two key factors, one was that we selected and isolated more higher metastatic subpopulation from the parent tumor, and the other was that a long time could give chance to implanted tumor to metastasize.

As shown in the results, the expression of AFP, MDR1, and LRP was well maintained in locally growing tumor tissues as well as in metastases. These results indicated that this nude mice model represented the majority of biological natures including local invasion, spontaneous lung, bone and intrahepatic metastases, MDR and secreted AFP, suggesting that our model indeed has some features resembling the natural biological behaviors of human HCC.

Although spontaneous metastases models of HCC have been established in nude mice, but bone metastases and MDR studies are not available. Our model well maintained the native structure of HCC and exhibited spontaneous lung metastases, especially spontaneous bone metastases and expression of MDR just as the patient donating the specimen. Thus, this model represents the features of patients with HCC, and could be an interesting tool for studying human HCC.

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