Current Medical Science
DOI https://doi.org/10.1007/s11596-019-2111-6
39(5):820-824,2019

Establishment of Rabbit Liver VX2 Tumor Model Using Percutaneous Puncture Inoculation of Tumor Fragment Guided and Evaluated by Ultrasonography

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Summary: The aim of the present study is to evaluate a method of establishing model of rabbit liver VX2 tumor using percutaneous puncture inoculation of tumor fragment guided by ultrasonography. VX2 tumor fragments were implanted into the liver of 13 New Zealand white rabbits flushed by 1 mL normal saline through percutaneous puncture needle guided by ultrasonography. Conventional ultrasonography and contrast-enhanced ultrasonography (CEUS) were performed 14 days after inoculation, and then the rabbits were sacrificed and pathologically examined. The success rate of inoculation was 100%. The average size of liver VX2 tumor was 1.7 cm×1.3 cm, CEUS of VX2 liver tumors showed the "rapid wash-in and wash-out" vascular pattern. There were significant differences between VX2 tumors and liver parenchyma in quantitative parameters of A, k and A × k (P<0.05), which meant that VX2 liver tumors were characterized by more blood flow volume and faster blood velocity than liver parenchyma. Tumor fragment flushed by normal saline into the liver through a needle may be a promising method for the induction of a hepatic tumor. And CEUS can be used for accurately assessing angiogenesis and blood perfusion of VX2 tumors.
Key words: liver; VX2 tumor; rabbit; ultrasonography; contrast-enhanced ultrasonography

Hepatocellular carcinoma (HCC) is one of the most common types of liver malignant tumor, and is considered as the third major cause of mortality from cancer worldwide[1]. It is demonstrated that surgical interventions (including complete resection and liver transplantation) are potentially curative treatments and offer the best prognosis for HCC[2]. Besides a number of potential and non-surgical treatments for HCC, such as transcatheter arterial chemoembolization (TACE), radiofrequency ablation (RFA) and high-intensity focused ultrasound (HIFU) therapy also developed widely[3, 4]. Thus, a suitable hepatic tumor animal model is important for the study of liver cancer diagnostics and various treatments[5].

VX2 carcinoma originated from Shope virus-induced human papilloma-derived squamous cell carcinoma and was formed after 72 transplantation passages[6]. Previous investigation has shown that the rabbit VX2 hepatic tumor grows rapidly, and its arterial blood supply is similar to that of human liver cancer and its size is large enough to be observed by clinical imaging[7]. Hence, the rabbit VX2 hepatic tumor model has been widely used to investigate various aspects of liver tumor behavior and is commonly recognized for the liver cancer inoculation[8, 9]. In previous studies, there are a number of different approaches to the induction of the VX2 tumor in the liver of rabbits including implantation of tumor cell suspension and tumor fragment suspension by laparotomy and percutaneous puncture. However, they still have deficiencies[10]. In the present study, VX2 tumor fragments will be implanted into the liver of rabbits flushed by 1 mL normal saline through percutaneous puncture needle guided by ultrasonography.

1 MATERIALS AND METHODS

1.1 Animals

This present study was approved by the Animal Use and Care Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and

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Technology (ID: TJ-C 20160456). All New Zealand white rabbits, aged from two to three months and weighing from 2 to 3 kg, were purchased from the Experimental Animal Center, Tongj Hospital (China).

1.2 Preparation of VX2 Tumor Fragments

Transplanted tumor of rabbit model was used to propagate and maintain the VX2 tumor. All rabbits were anesthetized with an intramuscular injection of ketamine injection (40 mg/kg, Sigma-Aldrich, Inc, USA) and xylazine injection (5 mg/kg, Sigma-Aldrich, Inc, USA). And 0.2 mL VX2 cell suspension was injected into the muscles of both hind limbs of the rabbits through fine needle. After three weeks of implantation, the tumor grew to a size of approximately 3 cm in diameter. Later, the rabbits were euthanized with an intravenous injection of sodium pentobarbital (100 mg/kg) and both hind limb tumors were obtained. The surrounding and necrotic tissues were removed from the tumor. Then, the tumor was cut into 1 mm^3 fragments, which were ready for inoculation[10, 11].

1.3 Implantation of VX2 Tumors into Liver

VX2 tumor fragments were implanted into the liver of 13 healthy New Zealand white rabbits. We took the sub-xiphoid area for the percutaneous puncture, penetrated a 16-guage needle into the left lobe parenchyma of rabbit liver guided by ultrasonography (fig. 1), and then pulled out the needle core, put a 1 mm^3 VX2 tumor fragment into the needle flushed by 1 mL normal saline, pulled out the needle and gently compressed the puncture place for 3 min after confirming the tumor fragment was rushed into the liver parenchyma (fig. 2).

1.4 Conventional and Contrast-enhanced Ultrasonography

Ultrasonography was carried out on day 14 after tumor inoculation. Each rabbit was fixed on an experiment table in the supine position. Then rabbits were anesthetized with ketamine and xylazine described above. Rabbit fur at inguinal regions and lower abdomen were removed for ultrasonic observation. Logic E9 (GE, USA) equipped with a 7 MHz harmonic-imaging transducer was used for contrast-enhanced ultrasonography (CEUS) and dynamic images recording. Firstly, conventional ultrasonography scan was performed to observe the tumors and identify the best tumor sections for CEUS. Thereafter, the SonoVue (Bracco Imaging, Italy), a second generation contrast agent composed of microbubbles of sulphur hexafluoride, was resolved in normal saline at a ratio of 1:5 and agitated for complete dissolution. Each rabbit underwent bolus injection of 0.3 mL SonoVue solution and then injected with 5 mL flush of 0.9% NaCl in bolus by the ear margin vein. A dynamic image of CEUS was acquired until the contrast agent diminished after contrast injection. All investigations were carried out in the same standardized method by the same ultrasound physician[11]. All dynamic images were reviewed by two ultrasound physicians and then the enhancement patterns of the tumors were recorded. Enhanced areas of the VX2 tumors were set as regions of interest (ROIs) to develop time-intensity curves and then parameters of perfusion analysis were quantitatively analyzed by software, by which quantitative parameters were generated from curve fitting formula: $F(t)=A(1-\exp[-kt])+B$, where A is the plateau value as an estimate of the regional blood volume, k is the replenishment rate as an estimate of microbubble velocity, and $A \times k$ is an estimate of flow, B is the baseline and t is the time[12].

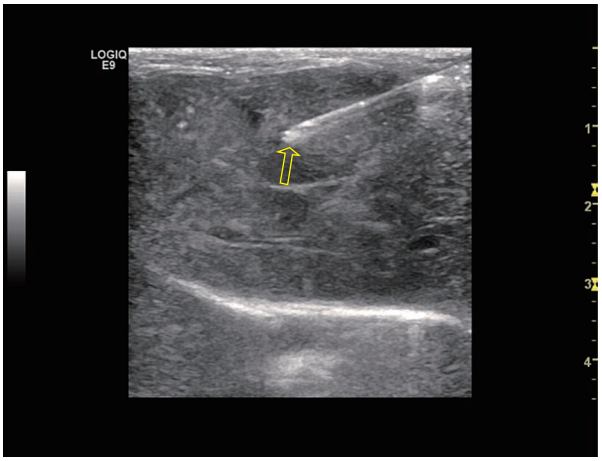

Fig. 1 A 16-gauge needle (arrow) was penetrated into the left lobe parenchyma of rabbit liver guided by ultrasonography

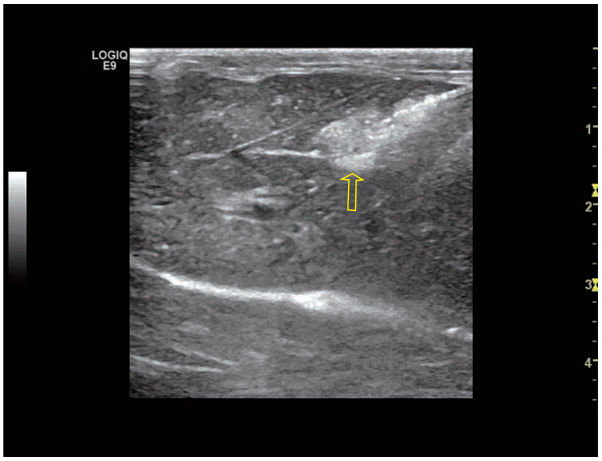

Fig. 2 The tumor fragment and 1 mL normal saline (arrow) rushed into the liver parenchyma showed heterogenous hyperechoic area

1.5 Histopathological Examination

Following completion of the CEUS, each rabbit was euthanized with an intravenous injection of sodium pentobarbital (100 mg/kg), and then the tumor growth in the liver was determined. All liver tumors were harvested for pathological examination by using hematoxylin-eosin (HE) staining.

1.6 Statistical Analyses

Statistical analysis was performed using SPSS

and a P value of <0.05 was considered to indicate statistically significant difference.

2 RESULTS

2.1 VX2 Tumor Formation in Rabbits

In the present study, all rabbits successfully tolerated VX2 tumor inoculation. No mortality and infection was observed during the implantation process. Of the 13 rabbits, all rabbits successfully tolerated VX2 tumor inoculation. Three rabbits had multiple lesions, and 10 rabbits had a single lesion. The average size of the tumors at the 14th day after inoculation was 1.7 cm×1.3 cm (table 1).

Table 1 The sizes of all rabbit liver tumors at 14 days after inoculation

No.	Tumor size (cm)	
	Major axis	Minor axis
1	1.6	1
2	1.5	1
3	1.6	1.9
4	1.4	1.2
5	1.8	1.4
6	1.6	0.9
7	2	1.6
8	1.7	1.1
9	1.5	0.9
10	2	1.5
11	1.9	1.5
12	1.7	1.2
13	2	1.4
Average	1.7	1.3

2.2 Conventional Ultrasonic Findings of VX2 Tumors

At 14 days after tumor inoculation, the rabbit liver tumors presented sphere or sphere-like homogeneous hypoechoic nodes with clear contour and without capsule echo. Color Doppler flow imaging showed blood flow signals in the peripheral regions and inside the VX2 tumors (fig. 3).

2.3 Contrast-enhanced Ultrasonography

CEUS showed significant peripheral enhancement of the VX2 tumors at the early arterial phase and then quick wash-out of contrast agents. The results of CEUS time-intensity curves of VX2 tumors also indicated the "rapid wash-in and wash-out" vascular pattern (fig. 4). There were significant differences between VX2 tumors and liver parenchyma in quantitative parameters of A, k and A × k, which meant that tumors were characterized by more blood flow volume and faster blood velocity than liver parenchyma (table 2).

2.4 Pathological Findings

Gross specimen of liver VX2 tumors showed pale and hard nodules without capsule in the liver (fig. 5). And histopathological image of the rabbit VX2 liver tumor showed irregular shaped and arranged tumor cells with large nucleus and enriched cytoplasm (fig. 6).

3 DISCUSSION

HCC is a form of highly malignant liver tumor, and its prognosis is poor[13]. It is crucial to find a suitable hepatic tumor animal model for investigations of HCC diagnostics and various treatments. VX2 carcinoma has been widely used to be an experimental model to investigate HCC diagnosis and treatment because of its hepatic arterial supply and rapid growth pattern[14].

It is considered that successful tumor inoculation and growth in the rabbit liver can lead to successful experimental investigations, so to some extent,

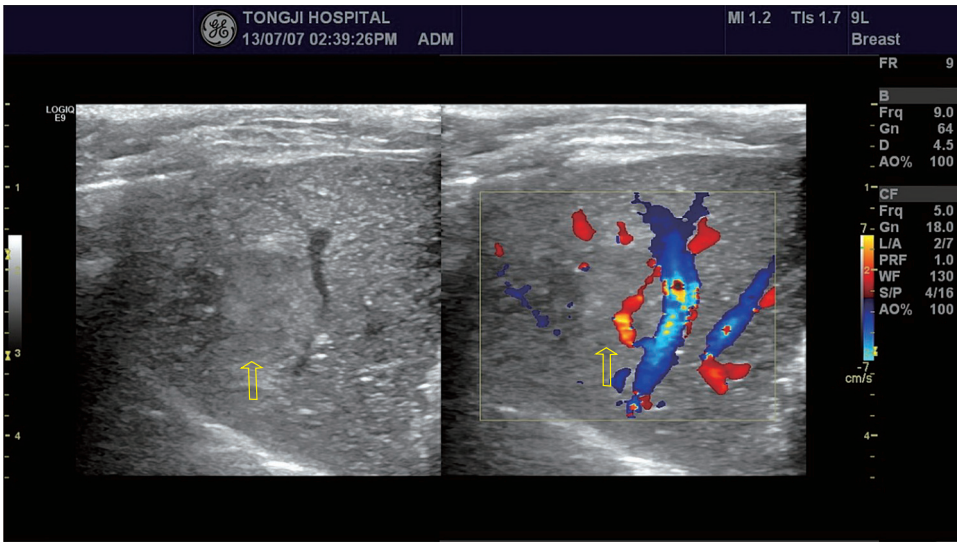

Fig. 3 Conventional ultrasonography showed that the rabbit liver VX2 tumor (arrows) at 14 days after tumor inoculation was a single sphere-alike homogeneous hypoechoic nodular with clear contour and there were blood flow signals in the peripheral regions as well as inside the VX2 tumor

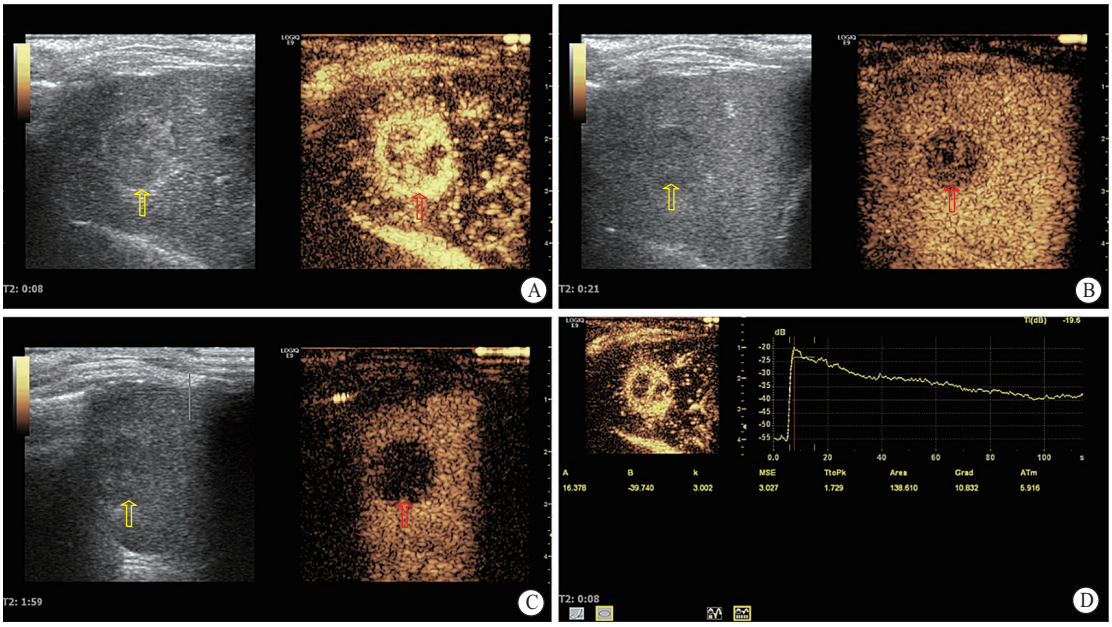

Fig. 4 Contrast-enhanced ultrasonography (CEUS) of the rabbit VX2 tumor (arrows)
A: CEUS showed heterogenous enhancement in arterial phase. B, C: CEUS showed that contrast agents were washed-out during portal and late phase. D: CEUS time-intensity curves and quantitative analysis showed the "rapid wash-in and wash-out" vascular pattern.

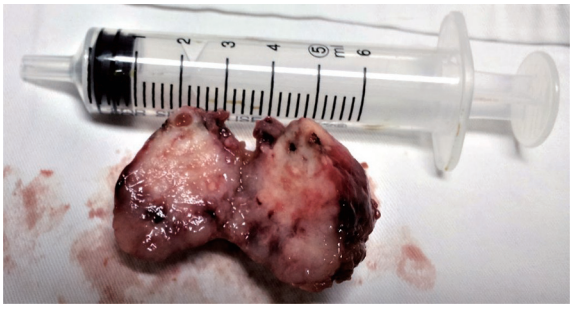

Fig. 5 Gross specimen of liver VX2 tumor showed a pale and hard nodule without capsule

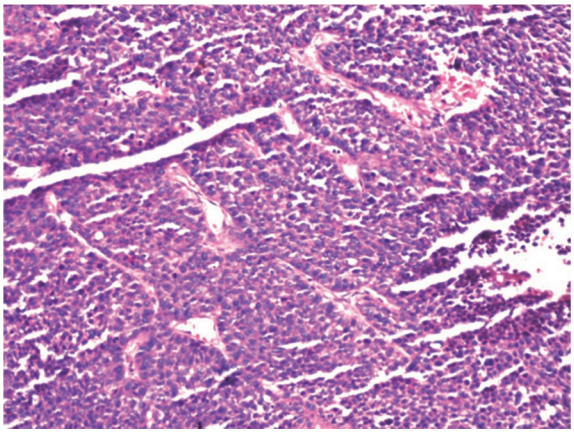

Fig. 6 Histopathological image of the rabbit VX2 liver tumor (hematoxylin and eosin-stained, ×200) showed irregular shaped and arranged tumor cells with large nucleus and enriched cytoplasm

continuous improvement of the tumor inoculation technique may improve the investigations of HCC.

Table 2 Parameters of CEUS quantitative analysis in rabbit liver VX2 tumors and liver parenchyma

No.	A		k		A × k	
	Tumor	Liver	Tumor	Liver	Tumor	Liver
1	32.307	22.716	1.472	0.316	47.556	7.178
2	30.033	23.044	1.337	0.46	40.154	10.6
3	36.233	30.394	0.859	0.392	31.123	11.914
4	40.083	39.399	1.502	0.835	60.205	32.898
5	35.426	32.388	0.919	0.772	32.556	25.003
6	37.784	31.245	0.201	0.323	7.595	10.092
7	30.385	25.543	0.607	0.312	18.444	7.969
8	25.338	27.554	0.411	0.192	10.414	5.29
9	25.174	31.26	0.388	0.258	9.767	8.065
10	23.53	29.986	0.567	0.548	13.341	16.432
11	30.893	25.079	0.899	0.61	27.773	15.298
12	29.902	16.561	0.476	0.719	14.233	11.907
13	25.814	21.892	0.559	0.235	14.43	5.144
	P=0.0479		P=0.0111		P=0.0059	

Previously, many investigators have achieved successful VX2 liver tumor inoculations by surgery approaches[15, 16]. In addition, it is reported that surgical inoculation of VX2 tumor can control the site of tumor growth more effectively, while tending to reduce the incidence of intra- and extra-haptic metastasis. However, there are also certain problems of the surgical tumor implantation. The surgical technique is more traumatic and surgical complications, including bile leakage, hemorrhage or abscess formation are more likely to occur[15]. In the present investigation, active VX2 tumor fragments were implanted into the liver of rabbits through percutaneous puncture guided by ultrasonography, and the excellent success rate and

formation of mainly solitary nodular rather than diffuse tumors are good for liver cancer imaging and treatment researches.

CEUS is a widespread imaging procedure for investigating the liver parenchyma. The injection of ultrasound contrast agents (USCA) and the use of specialized imaging techniques now allow to display tissue macro and micro-vascularization similarly to what is seen with contrast-enhanced computed tomography (CECT) or magnetic resonance imaging (CEMRI)[17]. Using CEUS, it was found that all VX2 tumors presented the same enhancement pattern: quick wash-in and wash-out. Enhancement in the peripheral regions of tumors was first shown and weak intratumoral enhancement was observed due to necrosis. Though the necrotic regions exist in tumor tissues, their acoustic impedance difference might not be large enough to be observed using B-mold ultrasound. Besides color Doppler flow and energy imaging are limited by spatial resolution, so they cannot fully detect flows in microvessels either. This minimal difference between necrosis and active tumor may be detected only by CEUS. And it revealed that the CEUS can reflect accurate perfusion situation of tumors.

In conclusion, tumor fragment flushed by normal saline into the liver through a needle, which is simple and effective, may be a promising method for the induction of a hepatic tumor. And CEUS can be used to accurately assess angiogenesis and blood perfusion of VX2 tumors. The results from our study may improve the efficiency of tumor inoculation.

Acknowledgments

Histopathological examinations were performed by the Department of Pathology at Tongji Hospital, Huazhong University of Science and Technology. The authors would like to acknowledge the Department of Pathology for providing these resources for this study.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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(Received Feb. 25, 2019; revised July 10, 2019)