

# Clinical Features and Clonal Origin of Diffuse Hepatocellular Carcinoma

Zheng-Lu Wang<sup>1</sup>, Lu-Zhou Zhang<sup>2</sup>, Yu-Liang Wang<sup>3</sup>, Wei-Ping Zheng<sup>4</sup>, Hong Zheng<sup>5</sup>

<sup>1</sup>Department of Pathology, Tianjin First Center Hospital, Tianjin 300192, China

<sup>2</sup>First Central Clinical College of Tianjin Medical University, Tianjin 300192, China

<sup>3</sup>Key Lab for Critical Care Medicine of the Ministry of Health, Tianjin 300192, China

<sup>4</sup>Tianjin Key Laboratory of Organ Transplantation, Tianjin 300192, China

<sup>5</sup>Organ Transplant Center, Tianjin First Center Hospital, Tianjin 300192, China

To the Editor: Diffuse hepatocellular carcinoma (D-HCC), also known as cirrhosis-like HCC, is a rare and peculiar type of HCC.<sup>[1]</sup> The World Health Organization (WHO) defines D-HCC as follows: small tumor nodules distributed diffusely all over the liver presents like cirrhotic-associated regenerative nodules.<sup>[2]</sup> Surgical resection, radiotherapy, and medication cannot bring good results for D-HCC, and the prognosis of D-HCC is poor. Besides, differentiating multicentric occurrence (MO) from intrahepatic metastasis (IM), multiomics profiling could provide essential information to evaluate the aggressiveness of existing lesions and apply personalized therapies as well as postsurgical treatment.<sup>[3]</sup> D-HCC occurs as an occult disease, providing few specific clinical manifestations, it resembles cirrhotic nodules on imaging, resulting in frequent misdiagnosis. Given the difficulty of obtaining tissues from the entire liver, recent research has focused on imaging studies.<sup>[2]</sup> The clonal origin pattern not only revealed the foundation of the tumor but is also closely associated with prognosis.<sup>[4]</sup> Therefore, this study aimed to determine the clonal origin pattern of D-HCC tumors and draw the conclusion concerning their clinical, pathological, imaging, and prognostic features. Jakate *et al.*<sup>[1]</sup> studied D-HCC livers obtained after transplantation. They summarized the pathological features of the disease and put forward a hypothesis for the clonal origin of the tumor; however, this hypothesis was yet to be proved.

Fourteen patients with D-HCC were enrolled for orthotopic liver transplantation from August 2004 to November 2010 in Tianjin First Center Hospital; D-HCC was diagnosed by postoperative pathology in accordance with the definition of the WHO. The following data were gathered: (1) sex, age, and disease background; (2) alpha-fetoprotein (AFP) concentrations before liver transplantation; and (3) imaging results before surgery. The exclusion criteria were as follows: (1) preoperative radiotherapy, antineoplastic drugs or interventional treatment was undertaken and (2) clinical, pathological, and follow-up information were not available. The aim of the study was to determine the clonal origin of the tumors through microsatellite loss of heterozygosity (LOH) tests. This type of HCC was further studied by integrating the clinical, pathological, and prognostic characteristics. The present

study was approved by the Ethics Committee of Tianjin First Center Hospital and confirmed to the ethical guidelines of the *Declaration of Helsinki*. The experiment used samples from the Biobank of Tianjin First Center Hospital. Informed consent was obtained from each patient. The procedure met all applicable guidelines of our institute as well as governmental regulations concerning the ethical use of donated organs.

Paraffin slices (10  $\mu$ m thick) were obtained for each tumor or nontumor tissue from the 14 cases. For tissue sampling, three pieces from the left and right lobes of the liver were taken randomly (sampling interval 3 cm). The tissues were extracted under a microdissection microscope, and then, the DNA was extracted following the instructions of the paraffin-embedded tissue DNA purification kit (Takara Biological Engineering Co., Ltd. China). The purity, concentration and RNA integrity number of the extracted DNA were then determined using an ultraviolet spectrophotometer (Agilent Bioanalyzer 2100). The DNA was considered pure if the *A260/A280* ratio was between 1.70 and 1.90, the RNA integrity number was 7–9, and the concentration was no <200  $\mu$ g/L.

Twelve high-frequency microsatellite LOH sites were studied in this research. The amplified products were then subjected to electrophoresis through an 8% denaturing polyacrylamide gel and silver stained. The polymerase chain reaction (PCR) and electrophoresis were conducted at least twice to ensure the accuracy of the test.

The number of molecules was the occurrence of LOH, and the denominator was the number of valid sites. When the single-strand conformation polymorphism-PCR results were compared between the tumor and nontumor tissues, LOH was determined if the intensity

**Address for correspondence:** Dr. Hong Zheng,  
Organ Transplant Center, Tianjin First Center Hospital,  
Tianjin 300192, China  
E-Mail: zhenghongxy@163.com

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**Received:** 11-12-2017 **Edited by:** Ning-Ning Wang  
**How to cite this article:** Wang ZL, Zhang LZ, Wang YL, Zheng WP, Zheng H. Clinical Features and Clonal Origin of Diffuse Hepatocellular Carcinoma. *Chin Med J* 2018;131:1128-31.

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10.4103/0366-6999.230725

of the band corresponding to the amplified product decreased by more than or equal to 50%, and retention of heterozygosity (ROH) was determined if the band intensity decreased by <50%. By comparing the LOH test results of different sites between tumors, the tumor was considered to have a multiclonal origin if the LOH was  $\geq 30\%$ , and it was considered as having a monoclonal origin IM if the LOH was the same and comprised all ROH. No decision was made about the clonal origin if the LOH was <30%.

Each diseased liver was cut parallel to the coronal plane with an interval of 0.5 cm, and the distribution and size of the tumors and cirrhosis on the section were observed and recorded. Three tissues were obtained from the left and right lobes of the liver randomly with an interval of 3 cm. Moreover, one piece of nontumor tissue from each lobe was obtained. The acquired tissues were fixed with neutral formalin and embedded in paraffin. The paraffin-embedded tissues were sectioned at 4  $\mu\text{m}$  in thickness and stained with hematoxylin and eosin. Two pathologists performed microscopic observations of the pathological slices in accordance with the WHO standards for the diagnosis, grading, and classification of HCC. They recorded the histological features, differentiation degree of the tumors, and microvascular tumor thrombus.

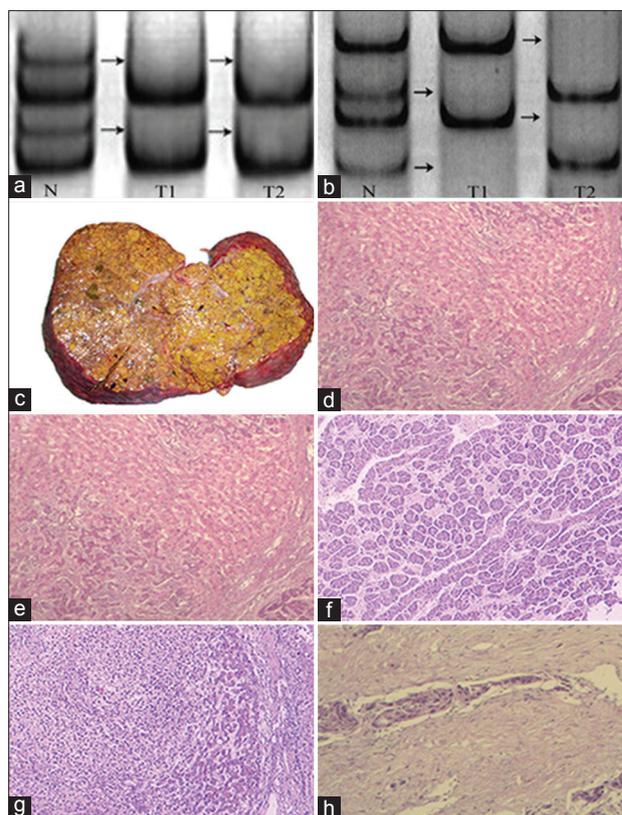
Follow-up for tumor recurrence ended 3 years after the liver transplantation. The tumor recurrence was identified using pathological checks. The information about the tumor-free survival periods of 14 patients was gathered.

SPSS 20.0 software (SPSS IBM, New York, USA) was applied to analyze the correlation between the clonal origin of the tumor and tumor recurrence.

Among the 14 patients with D-HCC in this study, 13 were males and one was female with an age range of 47–67 years (average 54 years). Their primary liver diseases included nine cases of hepatitis B virus (HBV)-related cirrhosis, three cases of hepatitis C virus (HCV) related cirrhosis, and two cases of simultaneous HCV- and HBV-related cirrhosis. Before surgery, the patients had no evidence of weight loss or abdominal pain. The ultrasound and computed tomography (CT) examinations showed similar images to cirrhosis, and their spleens were swollen. No clear tumor lesions were discovered before surgery. Eleven of the patients had enlarged livers. An enhanced CT imaging discovered no presentation of typical abnormal blood supply indicative HCC, no large-vessel embolus, and no lymphadenectasis. Five cases had serum AFP  $\leq 20$   $\mu\text{g/L}$ , five had serum AFP 20–200  $\mu\text{g/L}$ , and four had serum AFP  $>200$   $\mu\text{g/L}$ . The abnormal AFP was deemed to be induced by nonspecific or chronic active liver disease and conformed to the standards for liver transplantation.

LOH was observed in all 14 patients [Figure 1a and 1b]. The frequencies of LOH occurrence at 12 microsatellite sites are shown in Table 1. The results showed that the clonal origin of the tumors was the coexistence of MO and IM.

General presentation was as follows the slides of the livers had ashen, grayish yellow, and brownish green nodules. The boundaries of the nodules were clear, free from envelopes, and similar to cirrhotic nodules. More than 100 nodules were found. No emboli inside blood or bile vessels were observed. The histological presentation was as follows the HCC nests were distributed diffusely throughout the liver. The nests were surrounded by fibrous tissues [Figure 1c and 1d]. Between the nests, a scattering of cirrhotic and regenerative nodules was observed, some of which were invaded by cancer cells [Figure 1e]. The nuclei of the tumor cell were enlarged.



**Figure 1:** LOH patterns at microsatellite D16S514 in tumor 1 (T1), tumor 2 (T2), and normal tissue (N), Arrows show T1 and T2 LOH occur at the same site and different sites compared with noninformative (a and b); liver tissue section shows ashen, grayish yellow and brownish green nodules in a diffuse state (c); fibrous tissues surround HCC nests (H and E,  $\times 4$ ; d); Cancer cells invade cirrhotic nodules (H and E,  $\times 4$ ; e). Cancer cells in a trabecular array (H and E,  $\times 10$ ; f). Atypical hyperplasia of small hepatic cells inside the dysplastic nodules (H and E,  $\times 4$ ; g). A stromal vessel inside HCC thrombus (H and E,  $\times 20$ ; h). LOH: Loss of heterozygosity; HCC: Hepatocellular carcinoma.

Nucleolus and mitotic counting were performed, which showed that the cells were arrayed in a trabecular or false gland manner [Figure 1f]. The degree of tumor differentiation was largely Grade II with a few showing Grade I differentiation. In some nests, the tumor cell array and differentiation were matched. Dysplastic nodules were found in 12 patients during inspection, in which atypical hyperplasia of small hepatic cells was observed [Figure 1g]. Among all 14 patients, tumor thrombus inside the microvessels were found [Figure 1h]. After surgery, all 14 patients took the same immunosuppressive drugs, including tacrolimus, mycophenolate mofetil, and methylprednisolone. Their tumor-free survival period was 4.5–37.4 months, with an average of  $13.5 \pm 6.7$  months.

Only four cases had serum AFP  $>200$   $\mu\text{g/L}$  during the preoperative inspection in this study. Thus, AFP level should not be considered as a diagnostic marker; this was consistent with a previous study.<sup>[1]</sup> On imaging, D-HCC presented as diffusely distributed nodules in the liver,<sup>[2]</sup> which resembled cirrhotic nodules. The liver was also swollen. In some cases, a large-vessel embolus was found. The lack of space-occupying lesions, no large-vessel embolus or lymphadenectasis, and other imaging hints in the present study were similar to those in the previous reports. The data confirmed that imaging inspection also had

**Table 1: Frequencies of LOH\* at 12 microsatellite loci in D-HCC**

Patient number	D1S243	D1S507	D4S402	D4S406	D8S277	D8S520	D13S268	D16S419	D16S505
1	R	N	L	R	L	L	R	N	R
2	R	R	N	R	L	L	R	R	N
3	L	R	L	N	R	R	L	L	L
4	R	L	R	L	R	R	N	L	R
5	R	L	L	L	R	R	L	R	N
6	R	R	N	L	R	L	R	L	R
7	L	R	L	R	L	R	L	R	R
8	N	L	L	R	R	L	N	R	N
9	N	L	L	R	N	N	R	L	N
10	R	L	R	N	L	R	N	R	R
11	R	R	N	N	L	R	L	N	R
12	L	N	L	R	R	L	R	L	N
13	L	N	R	N	R	R	L	L	R
14	R	L	N	L	L	L	R	R	L

Patient number	D16S514	D17S831	D17S938	LOH, <i>n</i>	Different LOH, <i>n</i>	Informational sites, <i>n</i>	LOH proportion (%)	Clonal origin
1	L	R	N	4	3	9	33.3	IM + MO
2	L	R	L	4	3	10	30.0	IM + MO
3	R	R	N	5	3	10	30.0	IM + MO
4	R	L	R	4	4	11	36.4	IM + MO
5	N	R	N	4	3	9	33.3	IM + MO
6	L	R	N	4	4	10	40.0	IM + MO
7	R	L	R	5	4	12	33.3	IM + MO
8	R	R	L	4	3	9	33.3	IM + MO
9	L	R	L	5	3	8	37.5	IM + MO
10	L	L	N	4	3	9	33.3	IM + MO
11	L	L	R	4	4	9	44.4	IM + MO
12	R	R	L	5	3	10	30.0	IM + MO
13	R	L	N	4	3	9	33.3	IM + MO
14	R	L	R	6	4	11	36.4	IM + MO

\*LOH determination: Compared with the normal tissues, the density of the amplified product bands of tumor tissue DNA decreased by more than or equal to 50% or disappeared. D-HCC: Diffuse hepatocellular carcinoma; N: Noninformative; R: Retention of heterozygosity; L: LOH; LOH: Loss of heterozygosity; IM: Intrahepatic metastasis; MO: Multicentric occurrence; D-HCC: Diffuse hepatocellular carcinoma.

low sensitivity in the diagnosis of this disease. No large-vessel embolus or metastasis was found in the patients in this study, suggesting that they were relevant to the progress of the disease. This study confirmed the view that the combination of clinical characteristics, imaging, and AFP was not sufficient to make a specific diagnosis. Thus, if a cirrhosis patient has higher than normal AFP, the risk of D-HCC should be evaluated carefully, and a needle biopsy should be carried out if necessary for a correct diagnosis.

On visual inspection of the livers of the patients, the tumor nodules could not be distinguished from the cirrhotic nodules; it differed from the presentation of plurinodular HCC which matched the imaging characteristics.<sup>[4]</sup> Under the microscope, the HCC nests, cirrhotic nodules, and regenerative nodules were mixed. The tumor differentiation of the patients in this study was mainly the high type. Twelve of them had high-grade dysplastic nodules and small-cell dysplasia, which fitted the pathological characteristics of MO. The degree of tumor differentiation in the nests was synchronous, and the cancer cell invasion in the nests and the tumor thrombus in micro blood vessels were in consistent with the pathological characteristics of IM. The morphopathological characteristics of the patients in this study also conformed to the clonal origin pattern discovered herein.

Jakate *et al.*<sup>[1]</sup> proposed the clonal origin of D-HCC in their study; however, they have not yet confirmed it. Several methods may be used to determine the pattern of clonal origin of a liver tumor, including the microsatellite LOH test, p53 mutant-type detection, HBV-DNA integration detection, mitochondrial DNA methylation, and others.<sup>[5]</sup> Ng *et al.*<sup>[6]</sup> compared the merits and disadvantages of these methods and stated that the microsatellite LOH test had advantages such as wide applicability, convenience, local marker density, specific positioning, and low mutation frequency. Moreover, the microsatellite LOH test could be used for cancer tissue embedded in paraffin, which permitted review studies. Therefore, the microsatellite LOH test was used in this study to determine the clonal origin pattern of the tumors. Moreover, 12 high-frequency LOH sites were tested to increase the accuracy of the results. Furthermore, microdissection was used to obtain the cancerous tumor and nontumor tissues. The clonal origin patterns were determined as MO and IM, suggesting that D-HCC tumors occurred via MO accompanied by extensive IM. The clonal origin patterns of D-HCC tumor were very different from the plurinodular HCC, which had a tendency to show only a single pattern. Besides, IM was always included in D-HCC.<sup>[3]</sup> The mechanism of D-HCC occurrence requires further investigation; however, the existence of IM in the pattern of tumor clonal origin in D-HCC might explain the poor prognosis observed in clinical practice.

To date, the treatment of D-HCC has included radiotherapy, medication, and interventional therapy; however, they all have resulted in poor outcomes. If the tumors were diffusely distributed throughout the liver, surgical resection was not possible; liver transplantation becomes the only surgical option. In this study, 14 patients underwent liver transplantation and showed tumor recurrence within 1–2 years, accounting for 64.29% and 85.71% of the cases. This was not a satisfactory prognosis. However, three cases survived free from tumors for more than 3 years. Compared with the other methods, transplantation was relatively effective.<sup>[7]</sup> However, more multicenter studies are needed to confirm our results.

In conclusion, the D-HCC is an occult disease, the clinical markers and imaging inspections have low sensitivity. Therefore, the diagnosis of this disease is challenging. Moreover, D-HCC is invasive, and its prognosis is poor. Thus, future studies should strive to increase the diagnostic accuracy in its early stage, and more effective treatment strategies should be developed.

### Financial support and sponsorship

This study is supported by the grants from the Tianjin Clinical Research Center for Organ Transplantation Project (No. 15ZXLCSY00070), the National Human Genetic Resources Sharing Service Platform (No. YCZYPT[2017]02) and the Tianjin Health and Family Planning Commission Key Project (No. 13KG103).

### Conflicts of interest

There are no conflicts of interest.

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