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Clonality Analysis for Multicentric Origin and Intrahepatic Metastasis in Recurrent and Primary Hepatocellular Carcinoma

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Abstract
Aims To clarify the incidence of multicentric occurrence (MO) and intrahepatic metastasis (IM) for hepatocellular carcinoma (HCC) related to hepatitis B virus in China and to identify the differences between them.
Methods Histopathologic and genetic features of primary and recurrent tumors in 160 cases with HCC were analyzed. The two groups, the origin of which was definitely determinable as of multicentric occurrence or as of intrahepatic metastasis, were analyzed for their disease-free survival and clinicopathological differences.
Results According to histopathological findings, 27.5% and 59.4% patients were considered to be MO and IM, respectively. By comparing the genetic information of loss of heterozygosity and microsatellite instability for 10 different markers between primary and recurrent tumor, 30.0% and 63.8% patients with recurrent HCC were considered to be MO and IM, respectively. In total, 126 cases with unanimous conclusions from the histopathological and genetic method were selected and divided into the MO group (37 cases) and the IM group (89 cases). Analysis of stepwise regression identified that recurrence time, grading, portal vein invasion, tumor number, and Child's stage were the most important discriminating factors between MO and IM ($p<0.05$). As for their prognosis, Kaplan–Meier and log rank test showed that the disease-free survival in the MO group was significantly better than in the IM group ($p=0.002$).
Conclusions Combined analysis of histopathological and genetic analysis may reflect more exactly the nature of recurrent HCC. The incidence of MO in China is lower than in other countries—30% compared to up to 50% in Japan [Morimoto et al., *Journal of Hepatology* 39:215–221, 2003; Yamamoto et al., *Hepatology* 29;1446–1452, 1999]. Recurrence time, tumor grading, portal vein invasion, tumor number, and Child's stage are the most important discriminating factors between MO and IM. The prognosis (disease-free survival) of patients with MO compared to IM is significantly better.

Keywords Hepatocellular carcinoma · Recurrence · Multicentric occurrence · Intrahepatic metastasis · Loss of heterozygosity

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and is particularly prevalent

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in China.[1,2] The incidence of HCC is prone to increase dramatically over the next few decades due to high infection rates with hepatitis B virus (HBV) and hepatitis C virus (HCV), which are known to be intimately associated with HCC.[3,4] Besides liver transplantation, operation is another preferable effective treatment for this problematic disease at present. Although for cancers accessible by surgery, survival has greatly improved over the last years, the 5-year survival rate still remains as low as 47% after surgical resection.[5] This is much lower when compared to other gastro-intestinal cancers, e.g., gastric[6] and colonic cancer.[7] One of the main reasons for this is that the incidence of intrahepatic recurrence is extremely high, even after curative resection. Recurrence in the remnant liver has two different reasons: it may originate from intrahepatic metastasis (IM) and/or from multicentric occurrence (MO) also known as multicentric carcinogenesis, which is independent from the original primary tumor.

Discriminating them is very important not only for the study of hepatocarcinogenesis, but also for the determination of therapeutic strategies. Some groups have reported the incidence of MO in patients with HCC related to HCV as high as 50%. HCC with IM recurs earlier and has a poorer prognosis than that with MO.[8–11] Aggressive therapy may not be warranted in cases with IM, but in cases with MO, intervention should be taken within the limits of liver functional reserve.[12] Most of these reports refer to HCC related to HCV; however, the incidence and clinicopathologic features of HCC associated with HBV remain unclear.

The diagnosis of IM and MO is mainly based on histopathological findings as reported by the Liver Cancer Study Group of Japan with modifications,[8] but it is relatively subjective.[13] Previous studies had used the integration pattern of HBV-DNA, the X chromosome inactivation assay, and comparative genomic hybridization (CGH) as tumor markers of clone origins.[16–18] However, these methods have their limitations such as being applied only to HCC patients who have integrated HBV-DNA, female patients or expensive equipment and reagents.[14] Besides this, the test of HBV integration with southern blotting needs enough genome DNA. Microsatellite polymorphism, mainly including loss of heterozygosity (LOH) and microsatellite instability (MSI), is an important genetic feature in carcinogenesis. The test of LOH has been reported to be useful for clone discrimination of multiple HCC.[15] This is a simple and inexpensive method and can be applied in studies with large samples.

Materials and Methods

Patients and Samples

Informed consent was obtained from all the patients for the collection of liver specimens, and the study protocol was approved by the Ethics Committee of Tianjin Medical University. The clinical pathological data were collected as described in an earlier study by us.[5] Among the patients with recurrent HCC receiving repeat surgical resection in the Cancer Hospital of Tianjin Medical University, 160 cases were selected between 2001 and 2006 according to the following criteria: (1) diagnosis of HCC confirmed by pathology; (2) second hepatectomy; (3) incisal margins negative; (4) serum hepatitis B surface antigen (HBsAg) positive and hepatitis C virus antibody (anti-HCV) negative; and (5) complete clinicopathologic data of the case. Postoperatively, primary and recurrent HCC tissues as well as corresponding non-neoplastic liver tissue were stored at −80°C in a tissue bank.

Observation of Pathology

The recurrent and primary tumor sections of all patients were collected and the diagnosis of HCC was confirmed by two pathologists according to the diagnostic criteria of primary HCC.[16] The clone relations between recurrent and primary tumor nodules from every patient were determined in accordance with conventional histological criteria.[8]

PCR-based LOH and MSI Analysis

Genomic DNA was extracted from primary and recurrent tumor and non-neoplastic liver specimens by proteinase K/sodium dodecyl sulfate digestion followed by phenol/chloroform/isoamyl and alcohol extraction. It was resolved with sterile water and stored at −20°C.

Ten microsatellite markers on multiple chromosomes (1, 3, 8, 9, 13, 16, and 17) were selected for LOH analysis (Table 1) because of their high frequencies of LOH reported in HCC. These markers were amplified by polymerase chain reaction (PCR) kit (Tanaka Biotech, Japan) performed on PTC-240 (MJ, USA). Annealing temperatures were determined by Oligo software and are listed in Table 1. The PCR products were confirmed by 2% agarose gel electrophoresis.

Loss of heterozygosity (LOH) and MSI were detected by denaturing polyacrylamide gel electrophoresis (PAGE). Amplified DNA was mixed with formamide loading buffer (98% formamide, 1 mM EDTA, 0.025% bromophenol blue, and 0.05% xylen cyanol) and denatured for 5 min at 95°C.

Table 1 Microsatellite Markers for LOH Analysis

No	Markers	Primer sequence	Annealing temperature (°C)	Product (bp)
1	D1S214	3′-CCGAATGACAAGGTGAGACT-5′ 3′-AATGTTGTTTCCAAAGTGGC-5′	51	120–142
2	D1S2797	3′-ATCACATCACACACAATGACTGTGG-5′ 3′-TGTCCATTCAAAGGATTGGTCTC-5′	55	144–180
3	D3S3681	3′-GTGAGAACCATTTGGGGCAG-5′ 3′-GGCGAGCTATCTGTCAGGG-5′	53	210–246
4	D8S277	3′-GATTTGTCCTCATGCAGTGT-5′ 3′-ACATGTTATGTTTGAGAGGTCTG-5′	51	121
5	D9S199	3′-ACACATTCATACCATAGCAGAGG-5′ 3′-GGGGAAAGCATTCAGACTTT-5′	51	144
6	D13S170	3′-GATAAACACATAGGCACATGG-5′ 3′-CCTGCAGAATTGTGAGTAATG-5′	53	234
7	D16S3091	3′-GGGAGATAGCCTTAAACTTTCTTAC-5′ 3′-TGTTGCTAATAACACTAGGCCA-5′	52	115–129
8	D17S796	3′-AATGTGGTCCTTGAAATCCT-5′ 3′-TTACTAGGATCAAGGGGCAT-5′	53	234
9	D17S831	3′-CGCCTTTCCTCATACTCCAG-5′ 3′-GCCAGACGGGACTTGAATTA-5′	55	194–246
10	D17S938	3′-GGACAGAACATGGTTAAATAGC-5′ 3′-ATGCTGCCTCTCCCTACTTA-5′	52	145

LOH loss of heterozygosity

Then, the mixture was cooled immediately on ice and loaded onto a gel composed of 8% acrylamide (19:1 acrylamide/bisacrylamide), 90 mmol Tris (pH 8.3), 89 mmol borate, 2 mmol EDTA, 7 mol ultrapure urea, 1.6% ammonium persulfate (APS), and 5 μl *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED). Samples were electrophoresed at 50 V for 6 h, immersed in ethidium bromide and visualized by Chemidoc.XRS (Bio-Rad, USA).

Follow-up

All patients were followed up at the outpatient clinic every 3 months with measurement of the serum alpha-fetoprotein level and hepatic ultrasonography every 2–4 months from the date of initial treatment up to November 2007, or up to the time of their death. When recurrence was suspected, further evaluations were made by abdominal computed tomography (CT) scan, if necessary, by ultrasound-guided biopsy to confirm the diagnosis. Defined end point was non-survival. Patients who died of another disease were lost to follow-up, which, in total, were 14 (8.8%).

Statistics

The univariate analysis with Student's *t* test, the chi-square test, and Fisher's direct probability test helped us to reduce the number of study variables substantially. For the multivariate analysis, a stepwise regression model was used to identify the most important discriminating factors between two groups. The disease-free survival was calculated by method of Kaplan and Meier, and the differences in survival between them were compared using log-rank test. A p value <0.05 was considered significant.

Results

Clonality Analysis Based on Pathological Features

According to histological findings, 27.5% (44/160), 59.4% (95/160), and 7.5% (12/160) patients were considered to be MO for polyclonal origin, IM for intrahepatic metastases, and indeterminate group without definitive histological differentiation, respectively**.** Both MO and IM types of nodules were presented simultaneously in 5.6% cases (9/160).

Clonality Analysis Based on LOH and MSI

Compared to normal tissue of the same patient, a visually determined reduction of over 50% in allele intensity (allelic loss) was considered as LOH and emerging of additional

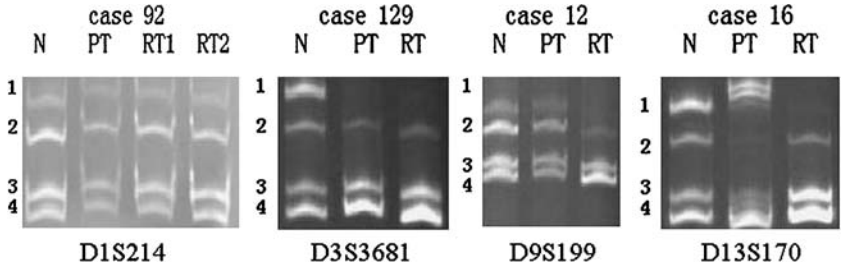

Figure 1 Case 92 showed no LOH and MSI in normal (*N*) tissue, primary tumor (*PT*), recurrent tumor (*RT*) 1 and *RT2* for marker *D1S214* (four bands presented at the same position). Case 129 showed LOH for marker *D3S3681* in *PT* and *RT* (no band 1 in *PT* and *RT* compared to that of *N*). Case 12 showed LOH for marker *D9S199* in *RT* (no band 1 in *RT* compared to that of *N*). Case 16 showed MSI (the positions of bands in *PT* were different from that of *N*) for marker *D13S170* and LOH (no band 1 in *RT* compared to that of *N*). *LOH* loss of heterozygosity, *MSI* microsatellite instability.

band(s) within a certain allele or a shift of an allelic signal was considered as MSI. The LOH pattern between the primary and recurrent tumors from one individual patient was regarded as identical when the same marker demonstrated loss of the same allele or no LOH (Fig. 1). It was regarded as different LOH pattern when the same marker demonstrated loss of one allele in either the primary or recurrent tumor but no loss or loss of the other allele in the other tumor. If the LOH patterns and MSI for the different markers reached 30%,[15] the recurrent nodule was considered of different clonality compared to the primary tumor (MO).

For all the 160 cases, the LOH for the 10 different markers ranged from 17.7% to 53.2% and the MSI from 3.8% to 15.2% (Table 2). In average, the LOH rate and MSI rate for the 10 markers was 35% and 10%, respectively. By comparing the genetic information of LOH and MSI between primary and recurrent tumor, 30.0% (48/160), 63.8% (102/160), and 3.8% (6/160) patients with recurrent HCC were considered to be MO, IM, and indeterminate ones due to insufficient information for some of the markers in the primary or recurrent HCC nodules, respectively. Because another four patients showed both MO and IM in the recurrent nodules, they were also not determinable.

Correlations Between Pathologic Features and Microsatellite Analysis and Grouping

Totally, the result concluded by pathologic features is significantly correlated to that demonstrated by analysis of microsatellite polymorphism (r=0.611, p<0.01). For all the cases where the analysis of clonality from the pathologic features and the microsatellite polymorphism was unanimous, it was possible to select and divide them into the MO and IM group for further study. In total, 126 patients qualified for this, 37 for the MO group, and 89 for the IM group. Thirty-four patients were excluded from further analysis since the origin of recurrent HCC could not be determined or both types were simultaneously present.

Clinicopathologic Features of MO and IM Groups

For further analysis between the two groups, the following clinicopathological variables were investigated: age, gender, Child's stage, platelet count, total bilirubin (TBIL), deconjugated bilirubin (DBIL), albumin, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), cholinesterase, cholesterol, tumor number (n<2 versus n≥2), location (recurrent tumor

Table 2 Rates of Heterozygosis, LOH and MSI for the 10 Markers in 160 Patients

Number	Marker	Heterozygosis (%)	LOH (%)	MSI (%)
1	D1S214	76.5	22.8	7.6
2	D1S2797	59.7	32.9	11.4
3	D3S3681	66.2	36.7	6.3
4	D8S277	82.0	40.5	3.8
5	D9S199	68.8	17.7	3.8
6	D13S170	77.6	49.4	8.7
7	D16S3091	70.4	58.2	10.1
8	D17S796	83.7	32.9	12.7
9	D17S831	79.4	25.3	15.2
10	D17S938	86.5	53.2	2.5

Heterozygosis: the alleles of homologous chromosome at the same site are different.

Table 3 The Clinicopathologic Features Between the Groups of MO and IM

Variables	MO group	IM group	*P* value
	n=37 (%)	*n*=89 (%)	
Age (years)	54.4±9.9	51.7±10.3	0.179
Gender (male vs. female)	31(84):6(16)	76(85):13(15)	0.818
Child stage (A, B and C)	14(38):23(62):0(0)	62(70):26(29):1(1)	0.002
Platelet count (10^9/L)	110±40	137±59	0.012
TBIL (umol/L)	14.9±8.3	16.0±9.9	0.560
DBIL (umol/L)	5.4±2.5	6.5±4.3	0.160
Albumin (g/L)	39.9±6.6	42.6±6.7	0.043
Globulin (g/L)	32.8±10.1	34.7±8.3	0.292
ALT (U/L)	57.5±36.6	48.9±33.5	0.206
AST (U/L)	44.3±38.2	52.7±31.6	0.246
ALP (U/L)	101.8±65.1	111.5±80.7	0.522
Cholinesterase (U/L)	6.4±2.8	7.6±3.2	0.047
Cholesterol (mmol/L)	5.9±4.1	6.5±5.2	0.522
Tumor number (*n*=1 vs. *n*≥2)	33(89):4(11)	54(61):35(39)	0.002
Location (same vs. different lobe)	17(46):20(54)	59(66):30(34)	0.033
Tumor size (cm)	2.87±1.46	3.01±1.81	0.673
Capsule (present vs. none)	13(35):24(65)	23(26):66(74)	0.293
Histological grading (1, 2, and 3)	15(41):19(51):3(8)	12(13):53(60):24(27)	0.001
Portal vein invasion (positive vs. none)	5(14):32(86)	36(40):53(60)	0.003
AFP≥100 vs. AFP<100 (ng/ml)	29(78):8(22)	63(71):26(29)	0.382
Recurrent time (months)	23.9±13.0	15.6±11.9	0.001

MO multicentric occurrence, *IM* intrahepatic metastasis, *TBIL* total bilirubin, *DBIL* direct bilirubin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *AFP* a-fetoprotein

same lobe versus different lobe compared to primary tumor), tumor size (primary tumor), capsule (present versus no capsule), histological grading (1:2:3), portal vein invasion (invasion versus no invasion), a-fetoprotein (AFP) (>100 ng/ml versus <100 ng/ml) and recurrent time. The following variables were significantly different between group MO and group IM by univariate analysis: Child's stage, platelet count, albumin, cholinesterase (host factors), tumor number, location (compared to the primary tumor), histological grading, positive portal vein invasion in primary tumor (primary HCC) and recurrent time (factors of recurrent HCC) (Table 3). Analysis of stepwise regression identified that recurrent time (months), grading, portal vein invasion, tumor number, and Child's stage were the most important discriminating factors between MO and IM ($p<0.05$; Table 4). As for their prognosis, Kaplan–Meier and log-rank test demonstrated the disease-free survival in group MO was significantly better than that in group IM ($p=0.002$) (Fig. 2).

Discussion

An accurate method to identify the origin of a recurrent tumor in an individual patient is to determine whether the recurrent tumor and primary one are monoclonal (intrahepatic metastasis, IM) or polyclonal (multicentric occurrence, MO). Distinction between them has conventionally been determined by pathological criteria. Though pathological observation is relatively subjective, it is still the most convenient method in distinguishing MO and IM. Our results based on pathology only showed that 27.5% (44/160) and 59.4% (95/160) patients were MO and IM, respectively. Moreover, in a certain number of cases no

Table 4 The Discriminating Factors Between Group Mo And Group IM By Stepwise Regression

Variables	β	Std. Error	*t*	*P* value
Recurrent time (month)	−0.018	0.004	−4.153	0.004
Grade	0.150	0.053	2.825	0.006
Portal vein invasion	0.179	0.074	2.420	0.017
Tumor number	0.163	0.075	2.168	0.032
Child's Stage	−0.147	0.069	−2.144	0.034

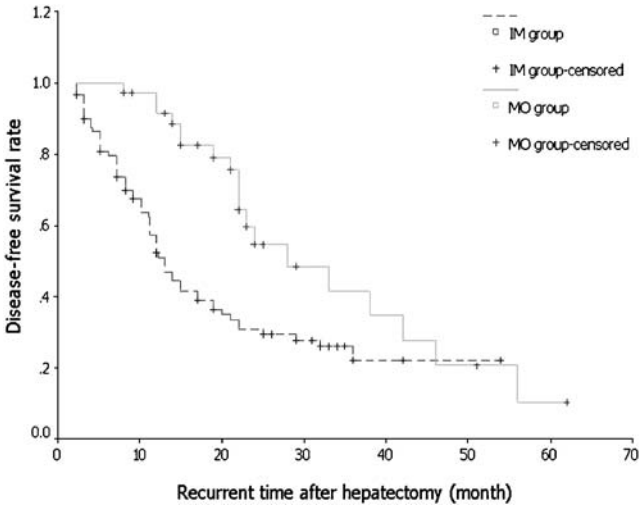

Figure 2 Kaplan–Meier and log rank test demonstrated the disease-free survival in group MO was significantly better than that in group IM (p=0.002). *MO* multicentric occurrence, *IM* intrahepatic metastatic. (Censored means mainly the cases without outcome of recurrence at the end of observation or the patients who were lost to follow-up.)

definitive differentiation between recurrent and primary tumor was possible, which suggested the limitation of this method when used alone.

The most precise and specific methods for assessing tumor clonality depend on the detection of common patterns of aberrations in DNA among the recurrent and primary tumors. Recent studies have indicated that in HCC, frequent aberrations are present in several genomic regions, including 1p, 4q, 5q, 6q, 8p, 8q, 10q, 11p, 13q, 16q, 17p, and 22q.[17–20] It has been suggested that an accumulation of these genetic changes, which affect the expression of oncogenes and tumor suppressor genes, occurs in a stepwise manner during HCC development and progression and can be used to identify the clonality of recurrent tumor. Therefore, in the present study 10 markers with a high frequency of LOH were selected to be amplified, which are located in seven different chromosomes. The extensive distribution may reflect more accurately the nature of the recurrent tumor than when just using markers for fewer chromosomes. With that, 30.0% (48/160) and 63.8% (102/160) of the patients with recurrent HCC were considered to be MO and IM, respectively. Besides LOH for the markers, we also noticed that MSI provides us valuable information in differentiating MO and IM, though its frequency in microsatellite polymorphism is much lower than that of LOH.

Compared with other molecular methods, the test of microsatellite polymorphism with PCR and PAGE showed many advantages as described before. Furthermore, it can be used for small quantities of genome DNA from tiny specimens such as fine-needle biopsy. This makes it possible to perform clone analysis for patients not qualifying for an operation. Meanwhile, it can also be used to study DNA fragments from paraffin-embedded specimen since the microsatellite markers are usually short DNA and can be amplified. In contrast, the test of HBV integration needs large quantities of genome DNA for Southern blotting and presents considerable limitation.

The combined analysis of pathological features and genetic data from LOH and MSI demonstrated that 23.1% (37/160) and 55.6% (89/160) were MO and IM, respectively. The percentage of MO is similar to that reported by Irene et al.,[21] but is less than that of most Japanese studies.[22,23] This may be caused by the different reasons for hepatitis. HCV is the most important risk factor in Japan. HBV, however, is intensively associated with HCC in China. It has been reported that the incidence of MO is much higher in HCV-positive patients than in HBV-positive ones.[24] The precise cause of this higher incidence of MO in HCV-positive patients is unclear. In general, however, it is well-known that cirrhosis due to HCV causes more severe and persistent active inflammation than cirrhosis originating from HBV.[25] Such persistent active inflammation may cause continuous necrosis and regeneration of hepatocytes; this could lead to DNA instability in the hepatocytes and could cause HCC to occur more frequently. Tarao et al.[26] studied DNA synthesis in hepatocytes in cirrhotic livers after hepatectomy for HCC. They reported that in 28 HCCs associated with HCV-related liver cirrhosis, a high labeling index was found in 14 HCCs, and nine of the 14 had recurrence (or new cancer) within 3 years after surgery. On the other hand, in the remaining 14 HCCs (which had a low labeling index) only three had recurrence in the same period. These findings suggested that accelerated hepatocyte regeneration seemed to be closely related to the occurrence of HCC. Their findings also supported the finding of a higher frequency of synchronous or metachronous multicentric occurrence of HCC in HCV-related liver cirrhosis in which persistent liver cell damage and regeneration of hepatocytes are common.[27]

In a further comparison between the MO group and the IM group, the discriminating factors include tumor grade, number, and portal vein invasion. However, without statistical significance, it appears that tumor size was noted to be smaller in the polyclonal group compared to the intrahepatic metastasis group. The different growth velocity may due to the different biological behavior in the two groups (cancer cells in IM showed more powerful invasion and metastasis than those of MO) and also presented in tumor capsule and location besides the three variables above. Meanwhile, the short intervals of follow-up counteract the proliferative dimensional significance in the two groups. As for the non-tumor factor, the distinct Child's stage suggests that liver cirrhosis in patients with MO was

more severe than in IM. It is believed to cause multiple premalignant and malignant nodules in the liver and is considered to be one of the most important factors of simultaneous and metachronous multicentric occurrence of HCCs.[28] The other factors, such as platelet count, albumin, globulin, and cholinesterase, also suggested the poor liver function reserve in the patients of group MO. Nevertheless, in our study the disease-free survival in the MO group is better than in the IM group. This demonstrates that in the determination of patients' prognosis, the biological behavior of a tumor plays a more important role than liver cirrhosis does. Although our study was confined to curative resected patients and excluded unresectable cases and led to some bias in the comparison of variables, the results were considered to be quite reasonable. As we know, many cases in both groups lose the opportunity of surgery because of various factors in which multiple tumors located in both liver lobes and severe liver cirrhosis (Child's C) are the most common reasons in group IM and group MO, respectively.

In conclusion, the combined analysis of pathology and test for microsatellite polymorphism shows much power in the determination of clone origin of recurrent HCC. The incidence of MO HCC was much lower than in Japan due to the different origin of hepatitis. Apart from the appraisal of recurrent time, tumor grade, portal vein invasion, tumor number, and Child's stage, the discrimination between MO and IM for recurrent HCC benefits the evaluation of patients' prognosis.

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