Dedifferentiation of Human Hepatocellular Carcinoma Up-regulates Telomerase and Ki-67 Expression

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Hypothesis: Dedifferentiation of human hepatocellular carcinoma (HCC) may influence telomerase activation and Ki-67 expression.

Design: Laboratory study using human HCC specimens.

Setting: University hospital.

Patients: Twelve patients with HCC with specific morphologic patterns (nodule-in-nodule [n=4] or confluent multinodular [n=8] type) and histological heterogeneity and who had undergone curative hepatectomy were studied. Of these, 8 patients had 2 different histological grades of HCC cells distributed at various nodules but within the same tumor; 3 patients, 3 different histological grades; and 1 patient, all 4 different histological grades.

Intervention: Tissue samples were retrieved from each nodule of the tumor and not mixed with one another. A total of 42 cancerous tissues from different distinctive nodules of the 12 patients were taken for telomerase and Ki-67 study, and corresponding noncancerous counterparts (n=12) served as healthy control samples. Telomerase activity was assayed by the telomerase repeat amplification protocol. Expression of messenger RNA (mRNA) by the human telomerase catalytic subunit human telomerase reverse transcriptase (hTERT) was determined using reverse transcription polymerase chain reaction. The relative telomerase activity and hTERT mRNA in each tissue sample was quantified using densitometry and expressed as a percentage of the standardized HeLa cell line. Immunostaining with anti–Ki-67 antibody was used to detect Ki-67 and was expressed as Ki-67 labeling index.

Main Outcome Measures: Telomerase activity, hTERT mRNA, and Ki-67 labeling index stratified by different histological gradings in each patient was analyzed. The correlations between telomerase activity and hTERT mRNA and between telomerase activity and expression of Ki-67 were plotted.

Results: Telomerase activity increased from more to less differentiated foci of HCC cells in each case (generalized linear model, P<.001). Mean ± SD expression of hTERT mRNA in 43 cancerous tissue samples, even those 4 with negative telomerase activity, was distinguishable from that of the noncancerous controls (0.84±0.23 vs 0.41±0.11; t test, P=.008). Telomerase activity was correlated to hTERT mRNA expression (Pearson correlation, r²=0.56; P<.001). The Ki-67 labeling index increased from more to less differentiated foci of HCC in each case (generalized linear model, P<.001). Expression of Ki-67 correlated with telomerase activity within differently graded areas within individual tumors (Pearson correlation, r²=0.38; P<.001).

Conclusion: Using the model of human HCC with histological heterogeneity, we determined that dedifferentiation of human HCC induces telomerase activation and Ki-67 expression.

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Human telomerase, a multicomponent ribonucleoprotein enzyme that extends chromosomal ends with (TTAGGG)n telomeric repeating sequences, is generally inactive in healthy human somatic cells but active in a variety of human tumor cell lines and primary malignant tissues. Downregulation of telomerase activity by induction of cellular differentiation has been reported for several cancer cell lines. Under physiologic conditions, undifferentiated cells display a much higher proliferative capacity than terminally differentiated cells in somatic tissues. Holt et al have shown that telomerase activity is present in the G1 (G1), synthesis (S), G2 (G2), and mitosis (M) phases of the cell cycle in dividing immortal cells, but not detected in cells that have exited the cell cycle. Taken together, the activation of telomerase might participate in controlling cell division and differentiation. Recent morphologic ex-

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MATERIALS AND METHODS

CLINICOPATHOLOGICAL DATA

We studied 12 eligible patients who had undergone surgical resection for primary HCC exhibiting distinct histological heterogeneity. The study group included 11 men and 1 woman, with a mean age of 54.3 years (range, 41-73 years). Hepatitis B surface antigen (HBsAg) and an antibody to hepatitis C virus (anti-HCV) were noted in 2 patients; HBsAg with no anti-HCV, in 7 patients; and anti-HCV with no HBsAg, in the remaining 3 patients. Liver cirrhosis was noted in 8 of the 12 patients, including macronodular (n=2), micronodular (n=3), and mixed (n=3) type. Abnormal α-fetoprotein levels (>20 µg/L) were noted in 7 of the 12 patients. Tumor size, in maximal diameter, ranged from 2.2 to 12.5 cm (mean, 5.5 cm). Grossly, the resected specimen could be categorized into the following 2 patterns: nodule-in-nodule (n=4), and confluent multinodular (n=8) type.6,7 Histological differentiation following 2 patterns: nodule-in-nodule (n=4), and confluent multinodular (n=8) type.6,7 Histological differentiation of the HCC cells was rated as grades 1, 2, 3, or 4, according to the criteria set forth by Edmondson and Steiner.8 Among the 12 patients, 6 patients had 2 different histological grades of HCC cells distributed at various nodules but within the same tumor; 3 patients, 3 different histological grades; and 1 patient, all 4 different histological grades.

TISSUE SAMPLES

The procedures for retrieving the tissue samples submitted for telomerase study were as follows. The gross specimens were bisected and photographed, and spatial distribution of the integrated tumorous nodules was plotted on a map and consecutively numbered. Tissues samples were taken from each nodule, with care not to mix them, and labeled. Thus, a total of 42 cancerous tissue samples from different distinctive nodules of the 12 patients were taken for telomerase study. Distinctive nodules within the same tumor did not necessarily have the different histological grades of HCC cells. The corresponding noncancerous counterparts (n=12), which were taken at least 3 cm from the cancerous part, if possible, served as healthy control samples. Each tissue sample was bisected, and one half was processed for routine histopathological examination and immunohistochemical study, and the other was frozen immediately in liquid nitrogen and stored at −80°C until use.

TELOMERIC REPEAT AMPLIFICATION PROTOCOL

The preparation of tissue extracts and the assay for telomerase activity by telomeric repeat amplification protocol (TRAP) have been described previously.9 Positive telomerase activity in an extract was determined by the presence of 6-nucleotide increment ladders of TRAP assay product in polyacrylamide gel electrophoresis that was sensitive to RNase A (Sigma-Aldrich Corp, St Louis, Mo) pretreatment. Extract from immortal HeLa cells was served as a positive control. Negative control was defined as reactions in the absence of extract protein. The gel was stained with syrhum bromide green DNA (Molecular Probe, Inc, Eugene, Ore), visualized, and photographed using illumination with a 254-nm UV light. We initially determined the intensity of the TRAP assay product using 0.05 to 5.00 µg of protein extract compared with that of the HeLa cells using 0.10 µg of protein extract, by means of computerized scanning densitometry (Image Quan; Molecular Dynamics, Chicago, Ill). According to our trials, TRAP assay using 1.0 µg of protein extract gave the best discrimination for the purpose of quantitation of the telomerase activity. The relative telomerase activity in each tissue sample was expressed as a percentage of that of the HeLa cell line. Relative telomerase activity lower than 10% and 1% were defined as negative and weakly positive, respectively.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Analysis of the expression of hTERT messenger RNA (mRNA) was performed using reverse transcription polymerase chain reaction (RT-PCR) amplification as described.9 The hTERT mRNA was amplified using the primer pair 5′-CAGAGATGTGCTGAAGCAAA-3′ (LT5) and 5′-GGATGAAGCGGAGTCTCTGA-3′ (LT6). The efficiency of complementary DNA (cDNA) synthesis from each sample was estimated by PCR with glyceraldehyde 3-phosphate-dehydrogenase (G3PDH)—specific primers of 5′-CTCAGACAATGAGGGAGTTGA-3′ (K136) and 5′-ATGATCTTGGAGCTTGTTCTTA-3′ (K137). Total RNA was isolated from the tissue with the use of a pre-extracted phenol and guanidium-isothiocyanate solution (Isogen; Nippon Gene, Tokyo, Japan) in accordance with the manufacturer’s protocol. The cDNA was synthesized from 1 µg of RNA using an RNA PCR kit (Version 2; TaKaRa, Tokyo, Japan) with random primers. To amplify the cDNA, 2-µL aliquots of reverse-transcribed cDNA were subjected to 28 cycles of PCR in 50 µL of 1× buffer (10-mmol/L Tris hydrochloride [pH, 8.3], 2.5-mmol/L magnesium chloride, and 50-mmol/L potassium chloride) containing 1 mmol/L each of deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxyadenosine triphosphate, 2.5 U of Taq DNA polymerase (TaqKaRa); and specific primers at a concentration of 0.2 mmol/L. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 90 seconds. The PCR products underwent electrophoresis in 2% agarose gel and stained with syrhum bromide green DNA. The hTERT RT-PCR products of each tissue sample were quantified using densitometry as described, normalized to the G3PDH RT-PCR signal, then compared with that of the standarized HeLa cell line.

IMMUNOHISTOCHEMICAL ANALYSIS FOR Ki-67

After deparaffinization, specimens were incubated with 0.1% trypsin in 37°C for 10 minutes and heated in a microwave 3 times for 5 minutes each at 750 W for antigen retrieval. Immunostaining was performed by means of the labeled streptavidin-biotin peroxidase method (Dako, Carpinteria, Calif) using an anti–Ki-67 antibody (MB-1; Immunotech, Marseilles, France) diluted 200-fold as the primary antibody.10 The Ki-67 labeling index was obtained by selecting equivalent fields at the margin, center, and midway between both areas in the HCC part. Areas around the portal and central veins and midway between both were evaluated in nontumorous controls. The Ki-67 labeling index represents the percentage of positive cells in at least 1000 cancer cells or hepatocytes.

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STATISTICS
Continuous variables were expressed as mean±SD. Difference of telomerase activity and Ki-67 labeling index in different histological foci of the same individual was examined using the generalized linear model followed by the Scheffé test. Linear regression and correlation between telomerase activity and hTERT mRNA were performed using the Pearson correlation. Linear regression and correlation between telomerase activity and Ki-67 were also performed using the Pearson correlation. Differences were considered statistically significant at P<.05.

RESULTS
Representative features of HCC exhibiting histological heterogeneity and the corresponding telomerase activity and hTERT mRNA expression are shown in Figure 1. Of 43 cancerous tissue samples taken from 12 patients, 39 (91%) had a remarkably detectable telomerase activity as determined by means of our nonradioisotope TRAP assay. When all the various histologically graded HCC cells of each case were accounted together, all 12 patients (100%) had positive telomerase activity in their cancerous tissues. In contrast, of the 12 noncancerous controls, 10 did not have detectable telomerase activity, and only 2 had weakly positive telomerase activity. The relationship of telomerase activity to histological grading in each case was plotted (Figure 2). Telomerase activity was increased from more to less differentiated foci of HCC in each case (P<.001, generalized linear model). In 2 cases with cytologic variants occurring in poorly differentiated cancerous tissue, clear cells in 1 case and spindle cells in the other, telomerase activity was 10-fold higher than that of more differentiated elements of the same subject. The mean ± SE level of the relative telomerase activity of 43 cancerous tissues, stratified by histological grades 1, 2, 3, and 4, were 20.0%±9.7%, 41.3%±20.2%, 75.6%±27.0%, and 96.6%±34.2%, respectively (grade 1 vs 2, P=.36; grade 1 vs 3, P=.002; grade 1 vs 4, P=.001; grade 2 vs 3, P=.05; grade 2 vs 4, P=.01; and grade 3 vs 4, P=.53, Scheffé test). The mean hTERT mRNA expressions of 43 cancerous tissues, even the 4 with negative telomerase activity, were distinguishable from those of noncancerous controls (0.84±0.23 vs 0.41±0.11, P=.008, t test). Linear regression and the correlation of telomerase activity and hTERT mRNA expression are shown in Figure 3 (Pearson correlation, r²=0.56; P<.001).

The representative features of Ki-67 staining for HCC detected using the monoclonal antibody is shown (Figure 4A). The Ki-67 labeling index was increased from more to less differentiated foci of HCC in each case (Figure 4B; P<.001, generalized linear model). The mean values of the Ki-67 labeling index, stratified by histological grades 1, 2, 3, and 4, were 30.4%±6.2%, 62.6%±13.2%, 79.6%±6.4%, and 87.0%±5.0%, respectively (grade 1 vs 2, P<.001; grade 1 vs 3, P<.001; grade 1 vs 4, P<.001; grade 2 vs 3, P<.001; grade 2 vs 4, P<.001; and grade 3 vs 4, P=.92, Scheffé test). The mean value of the Ki-67 labeling index of noncancerous controls was 33.3%±14.8%, which was lower than those of histological grade 2, 3, and 4 HCC cells (P<.001, Scheffé test) but was similar to that of histological grade 1 HCC cells (P>.99, Scheffé test). Furthermore, expression of Ki-67 correlates with telomerase activity within differently graded areas within individual tumors (Figure 5, Pearson correlation, r²=0.38; P<.001); however, this correlation does not exist in nontumorous parts.

COMMENT
Activation of telomerase has been investigated in HCC, chronic liver diseases, and precancerous hepatic nodules.11-16 To our knowledge, it has never been explored in regard to the telomerase expression of HCC cells that are undergoing histological and cytologic evolution. In 1997, Okuda et al17 first recognized that early HCCs were generally extremely well differentiated and suggested that subsequent histological changes toward more poorly differentiated cancer cells occurred as the tumor grew. Nakashio et al14 have shown that the order of telomerase activity is roughly well-differentiated HCC, then moderately differentiated HCC, and then poorly differentiated HCC, as did Kishimoto et al.15 However, reports by other investigators do not concur with this observation.11-13,16 The diversity of these reports might be attributed mainly to the fact that the HCC cells with different histological grades retrieved and tested for telomerase activity were not derived from the same individual. Furthermore, the existence of histological heterogeneity within the same tumor usually was not highlighted or was overlooked; hence, the tissue samples subjected to telomerase assay might have consisted of 2 or more histologically graded HCC cells, or they were not matched with the claimed histological grade. Lastly, the TRAP assay used in these studies was mostly semiquantitative by serial dilution, and the result was sometimes interfered with by the presence of telomerase inhibitor in more concentrated extraction. All of these pitfalls would result in
bias whenever measurement of telomerase expression, specifically stratified by histological grading, was attempted. In the present study, we tested telomerase expression in a cohort of patients with HCC associated with specialized confluent multinodular or nodule-in-nodule type. The advantages of this model are that most HCC cells retrieved from each nodule have the same histological grade, and the telomerase expression of various histologically graded HCC cells within the same individual can be titrated simultaneously, from which the kinetics of telomerase expression during dedifferentiation of HCC can be observed.

According to our results, telomerase activity progressively increases during the dedifferentiation of HCC in each individual (Figure 2). In 3 of our 12 patients, telomerase activity could hardly be detected in more differentiated foci, whereas it could be up-regulated maximally to 6.1- to 12.5-fold in the less differentiated foci of these subjects. Furthermore, cytologic variants of HCC cells, usually associated with poor histological differentiation, synergically affected the telomerase expression. Bile-producing HCC cells and glycogen-containing HCC cells (fatty change), representing a better-differentiated status, had a relatively low telomerase activity. In contrast, the poorly differentiated foci associated with cytologic variant, such as clear cells or spindle cells, had an extraordinarily high telomerase activity. Our data indicate that the process of dedifferentiation of human HCC cells, including histological and cytologic evolution, might up-regulate telomerase activity, from which the clinical observation that the less differentiated HCC cells behave biologically more aggressively can be explained.
Recently, hTERT has been identified as a putative human catalytic subunit. Accumulated data support the notion that the expression of hTERT is a rate-limiting step in the control of telomerase activity. Unlike telomerase activity detected by TRAP assay, varying from undetectable to remarkably high levels in cancerous tissues, the hTERT mRNA expression of cancerous tissues, even those with negative telomerase activity, was consistently distinguishable from those of noncancerous tissues (2-fold, at least). From this point of view, the probability of posttranscriptional modification of hTERT mRNA, which alters the enzymatic activity of telomerase, should be considered. Also, a difference in sensitivity may exist between TRAP assay and RT-PCR in detection of each target. Despite these differences, the titers of telomerase activity and hTERT mRNA expression generally showed a good correlation, as seen in Figure 3.

As a proliferating nuclear antigen, Ki-67 is present in cells that are replicating in the G1, S, G2, and M stages of the cell cycle. Since telomerase activity is also present throughout the G1, S, G2, and M phases of the cell cycle, Ki-67 seems to be a more optimal proliferating marker to reflect telomerase-active cells than proliferating cell nuclear antigen, which only accumulates in the late G1- and S-phase nuclei of proliferating cells. As seen in Figure 4, the Ki-67 labeling index of more differentiated HCC cells was lower than that of poorly differentiated HCC cells. This finding indicates that the proliferating capacity of HCC cells might be different in various histologically graded areas, even within the same individual, with the result that less differentiated cancerous areas tend to increase with tumor size, whereas well-differentiated cancerous areas diminish. Furthermore, expression of Ki-67 correlates with telomerase activity within differently graded areas within individual tumors, although this positive correlation is imperfect ($r^2 = 0.38$). In patients with underlying chronic persistent hepatitis, the Ki-67 labeling index in the noncancerous tissue could be as high as 60%, even higher than that of well-differentiated HCC cells. However, these highly proliferating hepatocytes, related to chronic persistent hepatitis, had only a weakly positive or undetectable telomerase activity, indicating a status far away from immortalization. Thus, proliferation alone is necessary but not sufficient for tumorigenesis.

In conclusion, by using the model of human HCC with histological heterogeneity, we determined that de-differentiation of human HCC up-regulates telomerase and Ki-67 expression.

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Short- and Long-term Survival After Cardiopulmonary Resuscitation
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Background: The objective was to evaluate the effect of patient characteristics and other factors on cardiopulmonary resuscitation (CPR) survival, hospital discharge survival and function, and long-term survival.

Methods: All patients 18 years and older experiencing in-hospital CPR from December 1983 through November 1991 at Marshfield Medical Center (Marshfield Clinic and adjoining St Joseph's Hospital), Marshfield, Wis, were selected. We performed a retrospective medical record review and augmented these data with updated vital status information.

Main Outcome Measures: Cardiopulmonary resuscitation survival, hospital discharge survival and function, and long-term survival.

Results: Of 948 admissions during which CPR was performed, 61.2% of patients survived the arrest and 32.2% survived to hospital discharge. Mechanism of arrest was the most important variable associated with hospital discharge. Patients with pulseless electrical activity had the worst chance of hospital discharge, followed by those with asystole and bradycardia. Follow-up information was available for 298 patients who survived to discharge. One year after hospital discharge, 24.5% of patients, regardless of age, had died. Survival was 18.5% at 7 years in those 70 years or older, compared with 45.4% in those aged 18 to 69 years. Heart rhythm at the time of arrest strongly influenced long-term survival. Bradyarrhythmias produced a nearly 2-fold increased mortality risk compared with normal sinus rhythm.

Conclusions: Survival until hospital discharge after CPR at our institution during an 8-year period was higher than previously reported for other institutions. Long-term survival after discharge was equal to or higher than reported estimates from other institutions. Hospital admission practices and selection of patients receiving CPR may account for these findings. (2000; 160:1969-1973)

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