Telomerase Activity in Precancerous Hepatic Nodules

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BACKGROUND. Recent studies have demonstrated that telomerase, a reverse transcriptase linked to cellular “immortalization,” is activated in a variety of malignant human tumors. This study was conducted to determine whether telomerase activity represents a marker of malignant transformation in precancerous (dysplastic) nodules arising in patients with cirrhosis.

METHODS. Telomerase activity was evaluated in frozen tissue samples of 14 cirrhotic liver specimens and 30 large nodular lesions contained therein, including 13 large regenerative nodules/large dysplastic nodules, 10 high grade dysplastic nodules, and 7 hepatocellular carcinomas (HCCs). A modified telomeric repeat amplification protocol was used.

RESULTS. There was a clear-cut difference in telomerase activity levels between HCC (positive or strongly positive) and cirrhotic liver samples (weakly positive or negative). The majority of large noncancerous nodules (86%) exhibited telomerase activity levels similar to HCCs. However, such activity was not limited to dysplastic lesions but also was detected in some large regenerative nodules.

CONCLUSIONS. These findings suggest that telomerase activation is an early event in large nodule formation in cirrhosis, which may facilitate the action of other factors in the process of carcinogenesis. Telomerase activity in large hepatic nodules is not always indicative of malignant transformation. Cancer 1998;82:1831–8. © 1998 American Cancer Society.

KEYWORDS: cancer, cirrhosis, dysplastic nodule, hepatocellular carcinoma, liver, precancerous lesion, regenerative nodule, telomerase activity.

The telomeres are special components of chromosomal ends containing repeated six nucleotide sequences, which protect the chromosomes from sticking to each other and undergoing structural changes.¹⁴ When normal somatic cells replicate, the telomeres are shortened, because DNA polymerases cannot finalize the copying process to the very end of the DNA strands. It has been proposed that decreasing telomere length over time signals cellular senescence or programmed cell death, thus protecting the genome from further changes that could lead to malignant transformation.

As opposed to normal cells, “immortalized” cells in culture and cancer cells in vivo exhibit short but stable telomere lengths, which are maintained by the action of telomerase, a reverse transcriptase with an RNA component.⁵⁻⁷ Recent studies have demonstrated significant telomerase activity in a variety of human carcinomas, including breast, liver, colon, prostate, and thyroid primary tumors.⁶,⁸⁻¹¹ Therefore, telomerase now is considered to be a potentially useful marker of malignant behavior in tumors of different organs.

Dysplastic nodules (DNs) recently have been identified as precancerous lesions arising in human livers with cirrhosis.¹²⁻¹⁹ These nodules can be detected on macroscopic examination because they stand out among the surrounding regenerative nodules by virtue of their
size, color, texture, or degree of bulging at the cut surface. Histologic examination is necessary for definitive identification of DNs. However, these lesions cover a broad spectrum of histologic appearances, ranging from features indistinguishable from those of large regenerative nodules to features reminiscent of well differentiated hepatocellular carcinoma (HCC). Differential diagnosis may be difficult or impossible at both ends of the spectrum, especially in small biopsy samples.18

We have hypothesized that activation of telomerase expression in precancerous nodules of cirrhotic livers may represent a marker of progression in the multistep process of carcinogenesis. We conducted this study to assess telomerase activity levels in precancerous lesions and to consider the possibility of using telomerase activity determination for diagnostic purposes. We followed the terminology for nodular hepatocellular lesions, which was proposed by an International Working Party that was sponsored by the 1994 World Congresses of Gastroenterology.19

MATERIALS AND METHODS
Tissues and Histologic Techniques
Fourteen cirrhotic liver specimens were selected from the files of the Mount Sinai Medical Center in New York City. Selection was based purely on availability of snap-frozen tissue samples of large nodular lesions. Thirteen specimens were livers removed on orthotopic transplantation, and one was a left lateral segment from the files of the Mount Sinai Medical Center in New York City. Selection was based purely on availability of snap-frozen tissue samples of large nodular lesions. Thirteen specimens were livers removed on orthotopic transplantation, and one was a left lateral segment resected for HCC. Each specimen was sectioned serially in 0.5 to 0.7-cm thick slices, and carefully examined for the presence of large nodules measuring at least 1 cm in greatest dimension and appearing distinct from the surrounding cirrhotic parenchyma in terms of color, texture, or degree of bulging at the cut surface. Fresh samples of all cirrhotic livers and 30 large nodules contained therein were snap-frozen on dry ice and stored at −70 °C until use. Additional sections from the same areas were submitted for routine histologic examination. Two normal liver samples also were snap-frozen and included in the study as “normal tissue controls.” These were derived from donor livers with essentially normal histology, which were not used for transplantation.

Four-micron thick, hematoxylin and eosin (H & E) stained sections of paraffin blocks were used for diagnostic classification, which was made into four groups: 1) cirrhotic liver; 2) large regenerative nodule (LRN)/low grade DN; 3) high grade DN; and 4) HCC. We followed the nomenclature and classification scheme proposed by the International Working Party15; however, we decided to combine LRNs and low grade DNs in one group because it is both this panel’s opinion and our own experience that microscopic examination cannot afford sufficient distinction between these two entities. The etiology of cirrhosis was determined on the basis of clinical, serologic, and histologic data.

Telomerase Activity Assay
Telomerase activity was assayed blindly (without knowledge of the histologic findings) by using the telomeric repeat amplification protocol (TRAP assay)6 with some modifications.20,21 Forty-six frozen samples, approximately 0.5 cm × 0.5 cm × 0.2 cm, were homogenized with a drill and disposable pestles in 100 μL ice-cold lysis buffer containing 0.5% CHAPS (Pierce), 10 mM Tris-HCl, 1 mM MgCl₂, 1 mM ethyleneglycoltetraacetic acid, 0.1 mM phenylmethyl-sulphonylfluoride, 5 mM 2′-mercaptoethanol and 10% glycerol. The samples were incubated on ice for 30 minutes and subsequently centrifuged at 14,000 revolutions per minute for 30 minutes (4 °C). Supernatant fluids then were transferred into new tubes and snap-frozen on dry ice. Protein content was measured by the BCA assay (Pierce), working dilutions of specimens were equalized to 3 mg of protein/mL, and the TRAP assay was performed using 6 μg of protein in each reaction. The initial TS-upstream primer extension step was followed by heating at 99 °C for 5 minutes to stop telomerase activity. Immediately thereafter, 2 U of Taq-polymerase (Boehringer-Mannheim Biochemicals, Indianapolis, IN), 2 μCi of [α-32P]-dCTP (Amersham, Arlington Heights, IL), and 0.1 μg of CX downstream primer were added to each tube in a polymerase chain reaction (PCR) mix containing 1× PCR buffer with MgCl₂. PCR was performed for 30 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 90 seconds. Fifteen microliters of the PCR product of each sample then was visualized by electrophoresis on 12% nondenaturing polyacrylamide gels followed by autoradiography at −80 °C for 8 and 48 hours (short and long exposure, respectively).

The reaction series was performed three times. For a semiquantitative assessment of telomerase activity, dilutions of 1×, 0.1×, and 0.01× were used (6.0, 0.6, and 0.06 μg of protein per reaction, respectively), as described in the literature.8 Specimens were evaluated as: 1) negative, when bands did not appear in any dilution; 2) weakly positive, when bands appeared after 48 hours’ exposure in the 1× diluted samples; 3) positive, when bands appeared after 8 hours’ exposure in the 1× samples; and 4) strongly positive, when bands appeared in the 0.1× or more diluted samples.

Lysis buffer was used as a negative control. True positivity was checked by adding RNase A into the samples.6,20 This enzyme cleaves the RNA component
TABLE 1
Patients’ Demographic Data, Etiology of Cirrhosis, Lesions Examined by the TRAP Assay and Results

| Case no. | Gender | Age (yrs) | Etiology of cirrhosis | CP | LRN/lgDN | hgDN | HCC |
|---------|--------|-----------|-----------------------|----|----------|------|-----|
| 1       | F      | 59        | Primary sclerosing cholangitis | wp | 2 p, p | 0    | 0   |
| 2       | F      | 57        | Alcoholic liver disease | wp | 0   | 2 p, p | 0   |
| 3       | M      | 51        | Unknown (cryptogenic) | wp | 0   | 0   | 2 p, p |
| 4       | M      | 59        | Chronic hepatitis B and delta | n  | 0   | 2 d, sp | 0   |
| 5       | F      | 20        | Chronic hepatitis C | wp | 1 p | 0   | 0   |
| 6       | M      | 56        | Chronic hepatitis C | n  | 1 wp | 0   | 0   |
| 7       | F      | 31        | Unknown (cryptogenic) | n  | 1 p | 0   | 0   |
| 8       | M      | 23        | Chronic hepatitis B | wp | 1 sp | 0   | 0   |
| 9       | M      | 55        | Chronic hepatitis C | d  | 0   | 0   | 1 sp |
| 10      | M      | 60        | Alcoholic liver disease | n  | 0   | 0   | 1 p |
| 11      | M      | 60        | Chronic hepatitis C-hemochromatosis | wp | 0   | 4 p, p | 1 sp wp |
| 12      | F      | 25        | Autoimmune hepatitis | n  | 6 p, p, p | 0 | 0 |
| 13      | M      | 70        | Fibropolycystic disease | wp | 0 | 0 | 1 p |
| 14      | F      | 47        | Chronic hepatitis B | wp | 1 p | 2 p, p | 1 sp |

TRAP: telomeric repeat amplification protocol; CP: cirrhotic parenchyma; LRN/lgDN: large regenerative nodule/low grade dysplastic nodule; hgDN: high grade dysplastic nodule; HCC: hepatocellular carcinoma; F: female; M: male; #: number of nodular lesions examined; *: TRAP results; d: degraded; n: negative; wp: weakly positive; p: positive; sp: strongly positive.

of telomerase, which then becomes inactive. A series of all ×1 samples were treated with 1 μg RNase A (Boehringer-Mannheim) each during the initial TS-primer extension step. To verify true negative results, 0.5 μL (1.5 μg protein) of a known positive specimen (from IMR-32, a neuroblastoma cell line) was added to the reactions as an “internal control,” as follows. All specimens were processed blindly at 1× and 0.1× dilutions, together with a parallel series of 0.1× diluted samples containing the “internal control.” In every case there was successful amplification of the “internal control”; thus, samples shown to be negative or weakly positive at the 1× and negative at the 0.1× dilution while simultaneously positive for the “internal control” were not processed further to the 0.01× dilution. Samples shown to be positive at the 1× and negative at the 0.1× dilution while positive for the “internal control” also were not processed further to the 0.01× dilution. Samples positive at the 0.1× dilution were processed to 0.01x, together with a parallel series containing the “internal control.”

RESULTS
Clinical Data and Pathologic Findings
Eight patients were men and six were women. Their ages ranged from 20–70 years (mean, 48 years). Demographic data, the etiology of cirrhosis, and classification of large nodular lesions are provided in Table 1. Overall, 13 nodules were found to be LRNs/low grade DNs, 10 high grade DNs, and 7 HCCs. Two LRNs/low grade DNs came from liver specimens with multiple (uncountable) large nodules (one each from Cases 5 and 8). All other lesions were derived from specimens with one to six large nodules in each. Six HCCs were well differentiated and one was moderately differentiated. All nodular lesions measured 1–2 cm in greatest dimension, with the exception of 3 cases of HCC (2 well differentiated and 1 moderately differentiated), which measured 3.8–5 cm.

On microscopic examination, the large noncancerous nodules were well circumscribed and surrounded by a rim of fibrous tissue, similar to that observed around cirrhotic nodules. LRNs/low grade DNs were comprised of normal or near-normal-appearing hepatocytes (Fig. 1A). Portal tracts were observed in a fairly regular distribution. In three lesions (one from Case 8 and two from Case 12), large cell change of hepatocytes was present. High grade DNs demonstrated evidence of cytologic or architectural atypia (Fig. 1B). Cytologic atypia, in the form of irregular nuclear contour, nuclear hyperchromasia, or increased nuclear-cytoplasmic ratio, was observed in all lesions, at least focally. Portal tracts were present in small numbers and irregular distribution. Architectural atypia in the form of a nodule-in-nodule growth pattern was observed in four lesions (one each from Cases 2 and 11 and two from Case 4), focal pseudogland formation in two lesions (one each from Cases 11 and 14), and focal unpaired (nontriadal) arteries in two lesions (one each from Cases 4 and 11).
FIGURE 1. Examples of large nodular lesions. (A) large regenerative nodule/low grade dysplastic nodule (Case 1). There is no evidence of cytologic or architectural atypia. Portal tracts are identified easily. The fibrous rim surrounding the lesion is observed at the lower end of the field. (B) High grade dysplastic nodule (Case 4). There is evidence of architectural atypia in the form of a nodule-in-nodule growth pattern. The majority of the field is occupied by a subnodule, which compresses adjacent liver cell plates. The hepatocytes are arranged in irregularly thick plates. (C) Well differentiated hepatocellular carcinoma (Case 3). The tumor cells are arranged in trabeculae of varying thickness. Pseudoglandular structures are identified easily (H & E, ×160).
The nodules of HCC lacked portal tracts, contained scattered unpaired arteries, and demonstrated more prominent cytologic atypia than DNs. Five of 6 well differentiated HCCs had trabecular architecture with pseudogland formation and bile production (Fig. 1C). In addition, one of these lesions (Case 9) showed dense hyalized collagen among the tumor cell trabeculae. The sixth well differentiated HCC (Case 10) featured thin, adenoma-like trabeculae. The single moderately differentiated HCC (Case 14) had trabecular architecture and included a large number of fat-containing tumor cells.

**Telomerase Activity Assay Findings**

The results of the TRAP assay are presented in Table 1. The assay was successfully performed in 44 of the 46 frozen tissue samples, with the appropriate controls ruling out false-positive and false-negative results. The assay was unsuccessful in one sample of cirrhotic liver and one sample of high grade DN, apparently because the tissues had degraded before processing. In these two cases, band-ladders were detected throughout the lanes and without regular distances among the bands (data not shown).

No telomerase activity was detected in the normal liver specimens. Five of 13 cirrhotic specimens were negative, and 8 exhibited weak activity. In the majority of large nodular lesions, including 10 of 13 LRNs/low grade DNs, 7 of 9 high grade DNs, and 4 of 7 HCCs, telomerase activity was positive. Two LRNs/low grade DNs were weakly positive, whereas another one, in a 23-year-old patient (Case 8), was strongly positive (Fig. 2A). Telomerase activity was weakly positive in one high grade DN, and strongly positive in another. Three HCCs also were strongly positive for telomerase activity. One (Case 14; moderately differentiated HCC) showed faint bands, even in the 0.01× dilution after 48 hours (Fig. 2B). A summary of telomerase activity in the various groups of lesions is provided in Figure 3.

**DISCUSSION**

In the last few years, the literature regarding telomere stabilization and telomerase activation in cancer has been growing rapidly, fueled to great extent by the hope of using telomerase activity as a marker of malignant behavior. Indeed, with the exception of germ cells and leukocytes, normal human cells do not express telomerase, whereas various malignant tumor cells do.\(^5\) Since 1995, eight studies,\(^6\) all from Japan, have assessed telomere length and telomerase activity in patients with chronic liver disease and HCC. As a rule, telomere length was found to decrease progressively from normal liver to chronic hepatitis to cirrhosis to HCC. In these studies, normal liver specimens lacked telomerase activity and specimens with chronic liver disease showed weak activity in 38–55% of cases, whereas HCC specimens, with few exceptions, were either positive or strongly positive for telomerase activity. Telomerase activity thus was proposed to be a useful marker for the early diagnosis of HCC.\(^26\)

In our study, there was no overlap in telomerase activity levels between specimens of cirrhotic parenchyma and those of HCC; none of the cirrhotic specimens was positive, and no HCC was either weakly positive or negative. These findings further support the widely accepted view that telomerase is activated at a high level in HCC. At this point, it should be noted that the level of positivity in our specimens generally appeared to be lower than that already described in HCC by other authors.\(^8\) This may be due to the modifications in the TRAP method we used, which included: 1) a higher temperature to stop the action of telomerase (99 °C vs. 90 °C); and 2) “hot start” PCR afterward, which made the amplification of the telomerase-extended TS primer products more specific. Nevertheless, our data are in good correlation with those of these previous studies.\(^8\) This is particularly evident in our case of moderately differentiated HCC, in which the strongest positivity was detected.\(^27\)

The focus of our study was the assessment of telomerase activity in precancerous hepatic nodules. Only three such nodules have been evaluated previously, and all showed telomerase activity of a level similar to HCC.\(^26\) We evaluated a total of 22 large non-cancerous nodules, 19 of which (86%) exhibited a degree of telomerase activity similar to HCC. The cirrhotic parenchyma surrounding the lesions was either negative or weakly positive for telomerase activity. These findings suggest that in hepatocarcinogenesis telomerase activation occurs before nodules with microscopic features of HCC are recognizable. A similar phenomenon recently has been described in a rat model of chemical carcinogenesis.\(^29\)

The terminology of nodular hepatocellular lesions recently has been standardized in the consensus article of the International Working Party.\(^1\) A review article that soon followed\(^30\) emphasized the distinguishing microscopic features of the various types of nodules. High grade DNs are differentiated from low grade DNs on the basis of cytologic or architectural atypia. The atypical features may either be diffuse or focal. Evidence of cytologic atypia includes irregular nuclear contour, nuclear hyperchromasia, increased nuclear-cytoplasmic ratio, cytoplasmic basophilia or clear cell change, rare mitotic figures, and resistance to iron accumulation. In high grade DNs, portal tracts are present in smaller numbers and more irregular distribu-
FIGURE 2. Representative telomeric repeat amplification protocol assay results, 48-hours exposure. (A) Lanes 1–5: Case 10. Negative cirrhotic parenchyma (CP) and a positive well differentiated hepatocellular carcinoma (HCC). Lanes 6–9: strongly positive large regenerative nodule/low grade dysplastic nodule (LG) from Case 8. (B) Lanes 2–13: Case 14. A strongly positive moderately differentiated HCC, two positive high grade dysplastic nodules (HG), one positive LG, and weakly positive cirrhotic parenchyma. The barely recognizable bands in Lane 4 (×0.01 dilution) were observed as faint bands in the autoradiogram. L: lysis buffer (negative control); ic: internal control.

FIGURE 3. Summary of telomerase activity in 44 liver samples. There is only minor overlap in telomerase activity levels between samples of cirrhotic liver and those of large nodular lesions. LRN: large regenerative nodule; DN: dysplastic nodule; HCC: hepatocellular carcinoma.
tion compared with low grade DNs; liver cell plates may be up to three cells in thickness; focal pseudogland formation may be observed; and expansile, clone-like populations of hepatocytes often are present that compress the adjacent liver cell plates or portal tracts in a nodule-in-nodule growth pattern.

HCC is distinguished from high grade DN on the basis of the following microscopic features\(^{19}\): absence of portal tracts, presence of stromal invasion, cell plates three or more cells in thickness, unattached “floating” cross sections of trabeculae, diffuse pseudoglandular structures, numerous unpaired arteries, moderately irregular nuclear contour, high nuclear-cytoplasmic ratio with significantly increased cell density, reduction of reticulin fibers, and more than rare mitotic figures. However, it has been emphasized that the features of malignancy may be focal, and differential diagnosis between HCC and high grade DN occasionally is impossible.\(^{19}\)

Separation of low grade DNs from LRNs is even more difficult. According to the International Working Party, the most certain way to distinguish between these two types of nodules will be the application of molecular genetic techniques, which is a prospect for the future.\(^{19}\) However, the following histologic features have been considered to favor a diagnosis of LRN over one of low grade DN: presence of a large (uncountable) number of lesions, presence of sinusoidal dilatation, and absence of cytologic atypia or clone-like features.\(^{18,19,30}\)

In our study, 9 of the 22 large noncancerous nodules assayed for telomerase activity were high grade DNs. Eight of 9 such nodules were either positive (n = 7), or strongly positive (n = 1) for telomerase activity. However, it is interesting to note that a similar incidence of positivity was detected in the category of LRNs/low grade DNs, with 11 of 13 lesions being either positive (n = 10), or strongly positive (n = 1). This category included lesions of either a regenerative (LRNs) or precancerous nature (low grade DNs). The high incidence of positivity in this category indicates that assessment of the telomerase activity level cannot be of help in distinguishing between these two types of nodules.

Although in the majority of LRNs/low grade DNs a regenerative versus precancerous nature could not be favored by microscopic examination, in two lesions there was other than histologic evidence of a regenerative character. These lesions were derived from liver explants from young patients (Case 5: age 20 years and Case 8: age 23 years), which contained multiple (uncountable) large nodules. Multiplicity of lesions and young age are believed to favor a regenerative rather than precancerous nature.\(^{18,30}\)

Telomerase activity was classified as positive and strongly positive in these two nodules, respectively. These findings indicate that significant telomerase activity may be detected in some lesions of regenerative nature. On the basis of current morphologic definitions of precancerous nodules,\(^{19}\) this is a substantial limitation to utilizing telomerase activity as a marker of malignant transformation in the liver.

The majority of DNs and LRNs (86%) exhibited telomerase activity levels similar to those of HCCs; however, the levels of three lesions were similar to those encountered in a subset of cirrhotic liver samples (weak positivity). The use of internal telomerase assay standards\(^{11,26,27}\) may provide more detailed information that could be useful in such areas of overlap, although true quantitation cannot yet be performed. Regenerating hepatocytes, small numbers of “immortalized” liver cells, and lymphocytes all have been proposed as possible sources of the weak telomerase activity observed in chronic liver disease.\(^{8}\) Nevertheless, weak telomerase activity in cirrhotic liver specimens should be considered to derive from a limited number of cells,\(^{27}\) which is unlikely to be the case in DNs. Further research in this direction is warranted; in situ TRAP assay may clarify the issue.\(^{31}\)

In conclusion, we found similar telomerase activity levels in HCC and the majority of large hepatic nodules arising in cirrhosis, including lesions with either a precancerous (DNs) or regenerative nature (LRNs). There was a substantial difference in telomerase activity levels between large nodular lesions and the surrounding cirrhotic parenchyma, although a minority of lesions exhibited overlapping levels. Our findings suggest that telomerase activation is an early event in large nodule formation in cirrhosis, which may facilitate the action of other factors in the process of carcinogenesis. However, telomerase activity cannot be used as a marker of malignant transformation. Although the majority of HCCs and precancerous nodules exhibit high telomerase activity levels, some large regenerative nodules also do.

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