Large Liver Cell Change in Hepatitis B Virus–Related Liver Cirrhosis

Haeryoung Kim,1 Bong-Kyeong Oh,2 Massimo Roncalli,3 Chanil Park,4 So-Mi Yoon,4 Jeong Eun Yoo,4 and Young Nyun Park4,5

Large liver cell change (LLCC) refers to microscopic lesions often found in various chronic liver diseases; however, its nature is still controversial. Thirty-four formalin-fixed and 19 fresh frozen hepatitis B virus (HBV)-related cirrhosis samples were examined for the presence of LLCC, small liver cell change (SLCC), and hepatocellular carcinoma (HCC). The cell cycle checkpoint status (p21, p27, p16, Tp53), cell dynamics (proliferating cell nuclear antigen, Ki-67, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling, M30), DNA damage (γ-H2AX [H2A histone family, member X]), telomere lengths, chromosomal instability (micronuclei index), and senescence-associated β-galactosidase (SA-β-Gal) activity were evaluated using an in situ approach and compared to those in normal liver (n = 5) and liver with chronic cholestasis (34 cases of hepatolithiasis and three cases of primary biliary cirrhosis). In HBV-related cirrhosis, the p21, p27, and p16 cell cycle checkpoint markers were activated in normal-looking cirrhotic hepatocytes (NLCH), but diminished gradually from LLCC, SLCC, to HCC, with an increase in Tp53 expression. There was a general decrease in telomere length from NLCH, LLCC, SLCC, to HCC. Micronuclei, γ-H2AX foci, and net cellular gain were significantly increased from normal hepatocytes, NLCH, LLCC, SLCC, to HCC. The SA-β-Gal activity was weaker in LLCC compared to NLCH and absent in SLCC and HCC. In contrast, cholestatic LLCC showed retained expression of cell cycle checkpoint markers and decreased net cellular gain compared to adjacent normal-looking hepatocytes. HBV-related LLCC showed significantly higher Tp53 labeling index, γ-H2AX labeling index, and micronuclei index; shorter telomere length; decreased SA-β-Gal activity; and increased net cellular gain compared to cholestatic LLCC. Conclusion: The nature of LLCC is rather heterogeneous depending on the biological setting. The characteristics of HBV-related LLCC are more consistent with dysplastic rather than merely reactive hepatocytes, whereas cholestatic LLCC more likely represents reactive change with more stringent cell cycle checkpoint control. (HEPATOLOGY 2009;50:752-762.)

Liver cell change (dysplasia) is characterized by individually scattered or clusters of hepatocytes with atypia, measuring <1 mm in diameter which do not form circumscribed nodules (dysplastic nodules), and have been often found in chronic liver disease.1,2 These lesions have been classified into two types: large liver cell change (LLCC) and small liver cell change (SLCC). SLCC, a lesion first proposed by Watanabe et al. in 1983,
is characterized by foci of crowded hepatocytes with high nuclear/cytoplasmic ratio, whereas LLCC is relatively easily recognized under the microscope as foci of cellular enlargement and nuclear pleomorphism, hyperchromasia, and multinucleation. Although SLCC has been more or less established as a precursor to hepatocellular carcinoma (HCC), the significance of LLCC is still controversial. Anthony et al., who coined the term “liver cell dysplasia” (now corresponding to LLCC) in 1973, found that LLCC was significantly prevalent in hepatitis B virus (HBV)-related cirrhotic livers harboring HCC and suggested that this lesion was preneoplastic. In addition, some studies recognized abnormal DNA contents (aneuploid peaks) and numerical chromosomal aberrations in LLCC, and increased net cellular gain in LLCC (higher proliferative index, lower apoptotic rates) compared to adjacent normal-looking cirrhotic hepatocytes (NLCH), supporting the hypothesis that LLCC may be a HCC precursor. Monoclonality has been found in macronodules with LLCC, and follow-up studies demonstrated that the presence of LLCC in HBV-related chronic liver disease significantly increased the relative risk of HCC development by three-fold to 16-fold and that LLCC had a high negative predictive value for HCC development. However, others disputed these findings by demonstrating LLCC had a low proliferative activity, high apoptotic rate, and no definite histologic continuum to HCC, and suggested that it may simply represent age-related/reactive change of hepatocytes.

The telomere is a TTAGGG-repeat sequence located at the end of the chromosome which is not replicated during the S phase of the cell cycle and, hence, is shortened after cell division. Loss of the telomere leads to repeated breakage-fusion-bridge cycles, chromosomal instability, and induction of a DNA damage response involving the cell cycle checkpoint pathways, leading to replicative senescence. In the absence of functional cell cycle checkpoint pathway responses, telomeres continue to shorten resulting in crisis. Neoplastic cells demonstrate high proliferative activity compared to normal cells, leading to accelerated cell cycles and shorter telomeres, and therefore require reactivation of telomerase before crisis for telomere length maintenance. The association between telomere shortening and chromosomal instability has also been demonstrated in HCC.

The DNA damage response induced by telomere dysfunction involves foci containing γ-H2AX (H2A histone family, member X), MRE11 (meiotic recombination 11 homolog), NBS1, MDC1 (mediator of DNA damage checkpoint 1), 53BP1 (p53 binding protein 1), RAD50, and BRCA1 (breast cancer 1, early onset). γ-H2AX is a phosphorylated histone H2A variant which facilitates DNA damage response by inducing changes in local chromatin structure and focal accumulation of DNA-repair and checkpoint proteins to damaged regions. Its presence is thus considered to reflect the accumulation of unrepaired DNA damage during aging. Another indicator of genomic instability in dividing cells are micronuclei, which are DNA masses in the cytoplasm of interphase cells arising from acentric chromosome fragments or whole lagging chromosomes in anaphase or telophase stages and appear similar to small nuclei. Micronucleated hepatocytes have been reported to be significantly more frequent in HCC compared to cirrhotic nodules and normal hepatic parenchyma.

Strong arguments indicate that replicative senescence is regarded as a tumor suppressor mechanism that prevents proliferation of genetically unstable precancerous cells, and involves cell cycle checkpoint activation and DNA repair foci recruitment. Therefore, activation of senescence-associated DNA damage checkpoints is ongoing in dysplastic lesions, but is gradually lost during malignant transformation.

Cyclin-dependent kinase inhibitors are important regulators of the cell cycle which inhibit unlimited cell growth by regulating G1/S progression. They have also been used as senescence markers; inactivation of both CIP/KIP and INK4a/ARF pathways have been shown to suppress cellular senescence in vitro. In addition, senescence-associated-β-galactosidase (SA-β-Gal), which can be detected at pH 6.0 only in senescent cells, and senescence-associated heterochromatin foci are other useful markers of senescence.

LLCC has been observed in various liver diseases including HBV and hepatitis C virus–related cirrhosis, autoimmune hepatitis, alcoholic cirrhosis, and chronic cholestatic disease. The various etiologies and contradictory reports for the nature of LLCC suggest that LLCC might be a heterogeneous lesion. Actually, the incidence of LLCC is higher in HBV-related chronic liver disease and it has been demonstrated that the presence of LLCC in liver biopsies of such patients is an important independent risk factor for hepatocarcinogenesis. To further characterize the nature of LLCC in HBV-related cirrhosis, we explored the molecular features—including senescence, cell cycle checkpoint status, DNA damage, and chromosomal instability—and cell dynamics of LLCC in HBV-related hepatocarcinogenesis, and compared them to those of LLCC in chronic cholestasis.

**Patients and Methods**

**Patient Selection.** A total of 34 surgically resected cases of HBV-related cirrhosis were selected for the study.
The patients ranged from 27-68 years (mean: 48 years) in age, and the male:female ratio was 2.4:1. The HBV-related etiology was confirmed by serological tests for hepatitis B surface antigen/hepatitis B e antigen or HBV DNA titer evaluation. Dysplastic nodules and HCCs were present in 11 (32.4%) and 21 (61.8%) cases, respectively. All specimens were formalin-fixed, paraffin-embedded, and cut into 4 \(\mu\)m-thick sections for hematoxylin-eosin and Feulgen staining, immunohistochemistry, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay, and quantitative fluorescent in situ hybridization (Q-FISH).

Fresh frozen liver tissue samples obtained randomly during initial gross inspection were available for SA-\(\beta\)-Gal study in 19 of the 34 HBV-related cirrhosis cases. This study was approved by the Institutional Review Board of Yonsei University Health System, and all specimens were supported by the Liver Cancer Specimen Bank from the National Research Resource Bank Program of the Korea Science and Engineering Foundation in the Ministry of Science and Technology.

For comparison, five histologically normal livers adjacent to metastatic colorectal carcinomas and 37 cases of chronic cholestatic livers (34 cases of hepatolithiasis with cholestasis, three cases of primary biliary cirrhosis) were selected. All cases were serologically negative for viral markers.

**Histological Examination.** All 34 cases demonstrated macronodular (\(n = 21\)) or macronodular and micronodular (\(n = 13\)) cirrhosis. The presence of LLCC and SLCC was determined according to previously described criteria: LLCC was defined as foci of hepatocytes showing cellular enlargement, nuclear pleomorphism, hyperchromasia, and multinucleation, whereas SLCC was defined as foci of crowded small hepatocytes with high nuclear/cytoplasmic ratio. LLCC was found multifocally in 31 of 34 (91.2%) cases, while SLCC was found in 19 of 34 (55.9%) cases (Fig. 1). LLCC and/or SLCC were present both in cirrhotic nodules and dysplastic nodules. Representative blocks containing at least one LLCC and/or SLCC focus were selected from each case for further analysis.

In cholestatic liver, LLCC was noted in 12 cases (35.3%) of hepatolithiasis and in all three cases of primary biliary cirrhosis. The five normal livers appeared normal on histologic analysis, without LLCC or SLCC. For comparison, representative blocks were selected from each case for further analysis.

**Immunohistochemical Stains for Markers of Cell Cycle, Proliferation, Apoptosis, and DNA Damage.** The antibodies used are summarized in Table 1. Sections were deparaffinized in xylene, rehydrated in graded alcohol, and quenched in 3% hydrogen peroxide. Antigen retrieval was performed as listed in Table 1, and incubation with primary antibodies was performed for 1 hour at room temperature. After rinsing, a secondary antibody (EnVision Rabbit/Mouse kit, DAKO, Glostrup, Denmark) was applied, and then developed with 3,3-diaminobenzidine. Slides were then counterstained with hematoxylin. Dark brown nuclear staining was counted as positive for all antibodies, and the labeling indices (LI) were determined for normal livers, cholestatic livers, normal-looking cirrhotic hepatocytes (NLCH), LLCC, SLCC, and HCC as follows: (number of positive hepatocytic nuclei in \(\times 400\) randomly selected fields)/(total number of hepatocytic nuclei) \(\times 100\%\).

**Q-FISH for Telomere Length.** Eighteen cases of HBV-related cirrhotic livers were subjected to telomere length analysis. The peptide nucleic acid probes used were as follows: Cy3-telomere probe (Applied Biosystems, Framingham, MA) and FAM-centromere probe (5’-FAM-OO-ATTCCGGTTGGAACGGGA-3’; Panagene, Daejon, South Korea). In brief, tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed in citrate buffer (pH 6.0)
Table 1. Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
<th>Antigen retrieval methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td>DAKO, Glostrup, Denmark</td>
<td>1:50</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
<tr>
<td>p27</td>
<td>DAKO, Glostrup, Denmark</td>
<td>1:50</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
<tr>
<td>p16</td>
<td>NeoMarkers, Fremont, CA</td>
<td>1:50</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
<tr>
<td>Tp53</td>
<td>DAKO, Glostrup, Denmark</td>
<td>1:50</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
<tr>
<td>PCNA</td>
<td>DAKO, Glostrup, Denmark</td>
<td>1:50</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>DAKO, Glostrup, Denmark</td>
<td>1:50</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
<tr>
<td>M30</td>
<td>Roche, Mannheim, Germany</td>
<td>1:200</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>Novus Biologicals, Littleton, CO</td>
<td>1:100</td>
<td>Microwave, citrate (pH 6.0)</td>
</tr>
</tbody>
</table>

in a 700 W microwave oven for 10 minutes, and sections were fixed in 10% buffered formalin. Sections were treated with protease I solution (1 mg/mL, Vysis, Downers Grove, IL) at 37°C for 10 minutes, dehydrated in graded alcohol, and air dried. The telomere/centromere probe mix (telomere: 2.5 μL 10 μg/mL PNA Cy3-telo- mere probe, 2.5 μL 25 μg/mL FAM-centromere probe) was applied, followed by denaturation at 80°C for 3 minutes and hybridization at 37°C for 2 hours using Vysis HYBrite. Sections were washed in post-hybridization buffer (NP40/20 × saline sodium citrate, Vysis) at room temperature for 30 minutes, air dried, and counterstained with 5 μL 4,6-diamidino-2-phenylindole (DAPI) I (Vysis). Sections were viewed under a fluorescent microscope, and photographs were taken from each lesion under 1000 magnification at the following exposures: 1/100 second for DAPI, and 1/3.5 second for Cy3 and FAM. The telomere fluorescence intensity (TFI) and centrome- re fluorescence intensity (CFI) were analyzed using Image Pro Plus 5.0 software (MediaCybernetics, Silver Spring, MD), and the mean TFI/CFI ratio were calculated for each lesion. The same study was performed for normal and cholestatic livers.

**TUNEL Assay for Apoptosis Detection.** The TUNEL assay was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International), according to the manufacturer’s instructions. The TUNEL-labeling index (TUNEL-LI) was calculated as the number of positive hepatocytic nuclei under ×400 magnification in at least five randomly selected fields by the total number of hepatocytic nuclei.

**SA-β-Gal Study.** Nineteen cases of fresh frozen HBV-related cirrhotic liver samples were cut into 6 μm-thick sections and mounted onto glass slides, fixed in 2% glutaraldehyde and 3% formaldehyde for 3-5 minutes and washed in PBS at room temperature. Sections were incubated overnight at 37°C with fresh SA-β-Gal stain solution (1 mg 5-bromo-4-chloro-3-indolyl-β-D-galac-toside [X-Gal]/mL [stock: 20 mg/mL in dimethylformamide] in 49 mM citric acid, sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricya-
increased slightly in HCC (Figs. 1 and 2; Table 2). The differences in p21-LI were statistically significant between NLCH, SLCC, and HCC and between LLCC, SLCC, and HCC. The p21-LI were lower in LLCC compared to NLCH, although not statistically significant. The p21-LI of normal hepatocytes was significantly lower than NLCH ($P < 0.001$).

Significant differences in p27 and p16-LI were found between NLCH and LLCC, SLCC, and HCC, and between LLCC and SLCC and HCC. The difference between SLCC and HCC was not significant for both p27 and p16-LI. The differences in p27 and p16 expression between normal hepatocytes and NLCH were statistically significant ($P < 0.001$, both).

Conversely, Tp53 expression increased from NLCH, LLCC, SLCC, and HCC. The differences in Tp53-LI were significant between NLCH and LLCC, SLCC, and HCC ($P < 0.001$, all); between LLCC and HCC; and between SLCC and HCC. The Tp53-LI between normal hepatocytes and NLCH was not significantly different.

**DNA Damage Markers in HBV-Related Hepatocarcinogenesis.** The $\gamma$-H2AX-LI increased from normal hepatocytes, NLCH, LLCC, SLCC, to HCC (Figs. 3 and 4; Table 2). The differences in $\gamma$-H2AX-LI were statistically significant between all lesions and also between normal hepatocytes and NLCH ($P = 0.002$). The micronuclei index was evaluated for 19 cases, and a similar increase in the same order was seen (Figs. 3 and 4; Table 2). There were statistically significant differences in the micronuclei index between all four lesions. Micronuclei were absent in normal hepatocytes.

**Proliferation and Apoptosis in HBV-Related Hepatocarcinogenesis.** The cell dynamics were studied, using proliferating cell nuclear antigen (PCNA-LI) and Ki-67-LI for proliferation and TUNEL-LI and M30-LI for apoptosis (Fig. 5; Table 2). An increase in proliferative activity (PCNA-LI, Ki-67-LI), was seen from normal hepatocytes, NLCH, LLCC, SLCC, to HCC, although the Ki-67-LI were, as expected, much lower as a whole. A significant decrease in apoptotic activity in LLCC compared to NLCH and increased apoptosis in HCC were seen ($P < 0.001$, both). The net cellular gain was then calculated by subtracting the apoptotic index from the proliferation index. When the TUNEL-LI was subtracted from the PCNA-LI, there was a significant increase in net cellular gain from NLCH to LLCC ($P = 0.010$) and a marked increase in HCC reaching as high as 94.94%. Similar patterns were shown by subtracting the TUNEL-LI from Ki-67-LI: statistically significant differences were found between NLCH and LLCC ($P =$
0.001), SLCC, and HCC. Subtraction of M30-LI from the two proliferation indices also demonstrated similar results.

**Telomere Lengths and SA-β-Gal Activity in HBV-Related Hepatocarcinogenesis.** The mean telomere lengths for LLCC, SLCC, and HCC were all significantly shorter than hepatocytes in NLCH (Fig. 6; Table 2). There was a gradual decrease in telomere length from NLCH, LLCC, SLCC, to HCC, with significant shortening in LLCC, SLCC, and HCC compared to NLCH. There was significant shortening of telomere length in NLCH compared to normal hepatocytes (P < 0.010). No significant correlation was seen between telomere length and patient age.

SA-β-Gal activity was seen in all 19 cirrhotic livers. LLCC was recognized in 17 of 19 cases; 2 of 17 (11.8%) LLCC showed weak SA-β-Gal activity, whereas SA-β-Gal activity was absent in SLCC and HCC (Fig. 7; Table 2). There were significant differences in SA-β-Gal activity between NLCH and LLCC and SLCC (P < 0.001, both).

**Comparison Between LLCC in HBV-Related Cirrhosis and Chronic Cholestatic Livers.** Detailed results are presented in Table 3 and Fig. 8. LLCC in chronic cholestatic livers (hepatolithiasis or primary biliary cirrhosis) showed higher p16-LI (P = 0.021) and decreased net...
cellular gain (Ki-67–LI – TUNEL-LI and Ki-67 – LI – M30-LI; P = 0.029 and P = 0.021, respectively) compared to adjacent normal-looking hepatocytes. Other cell cycle checkpoint markers (p21, p27) were similarly expressed in cholestatic LLCC and normal-looking hepatocytes. No significant differences in γ-H2AX–LI, micronuclei index, PCNA-LI, Ki-67–LI, TUNEL-LI, M30-LI, SA-β-Gal activity, and telomere length were seen between LLCC and normal-looking hepatocytes in cholestasis, and none of them showed Tp53 expression.

When LLCC in HBV-related cirrhosis was compared with those in chronic cholestasis, HBV-related LLCC showed significantly higher Tp53-LI, γ-H2AX–LI, and micronuclei index (P < 0.001, all). The telomere length was significantly shorter (P < 0.001), and SA-β-Gal activity was markedly decreased (P = 0.017) in HBV-related LLCC than in cholestatic LLCC. The PCNA-LI was higher (P < 0.001), whereas both TUNEL-LI and M30-LI were lower in HBV-related LLCC than in cholestatic LLCC (P < 0.001 and P = 0.033, respectively); therefore, HBV-related LLCC showed higher net cellular gain (Ki-67–LI – TUNEL-LI, Ki-67–LI – M30-LI) compared to cholestatic LLCC (P < 0.001, both).

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**Fig. 4.** Box plots showing frequency of (A) γ-H2AX foci and (B) micronuclei index in HBV-related hepatocarcinogenesis. Both indices increase significantly from NLCH, to LLCC, SLCC, and HCC.

**Fig. 5.** Box plots showing proliferation, apoptosis, and net cellular gain in HBV-related hepatocarcinogenesis. Remarkable increases in (A) PCNA and (B) Ki-67-LI are seen from NLCH to LLCC, SLCC, and HCC. (C) The TUNEL-LI decreases significantly in LLCC compared to NLCH and increases in SLCC and HCC. (D-F) The net cellular gain (PCNA-LI – TUNEL-LI, Ki-67-LI – TUNEL-LI and PCNA-LI – M30-LI) is significantly increased from NLCH to LLCC, SLCC, and HCC.
Discussion

Although LLCC is frequently found in cirrhosis and easily recognized even under low-power magnification due to the characteristic cytological features, its presence is rarely reported by pathologists in practice, and its significance is still under debate. Although some have favored a reactive/degenerative nature for the lesion, there is increasing evidence that it may actually be related to hepatocarcinogenesis. A comprehensive analysis of LLCC in HBV-related hepatocarcinogenesis was performed in this study, evaluating various aspects of the lesion, including the cell cycle dynamics, proliferation and apoptosis, DNA damage, and senescence.

The p21, p27, and p16 cell cycle checkpoint markers were expressed at the highest levels in NLCH but demonstrated increasing degrees of inactivation in LLCC, SLCC, and HCC, along with an increase in mutant Tp53 protein expression in HBV-related hepatocarcinogenesis. Plentz et al. previously reported that although the telomere was shortened in NLCH and significant further shortening was noted in LLCC, SLCC, and HCC, the cell cycle checkpoint marker p21 was intact in LLCC, only to be inactivated in SLCC and HCC, implying that intact checkpoint responses may prevent proliferation of LLCC with shortened telomeres and prevent the evolution of DNA damage and chromosomal instability. In this study, the expression of p27, p16, and Tp53 were also evaluated. The p21, p53, p27, and p16 markers are all cell cycle regulators involved in replicative senescence; p21 and p27 are components of the CIP/KIP pathway which are activated by p53 and transforming growth factor-β, respectively, and p16 is involved in the INK4a/ARF pathway. Both pathways result in inactivation of cyclin-dependent kinases and cell cycle arrest. Although the decrease in p21-LI in LLCC compared to NLCH was not statistically significant, the p27 and p16-LI were significantly decreased together with an increased expression of Tp53 in LLCC, implying that cell cycle checkpoint responses may actually already be partly inactivated in LLCC.

The increased expression of Tp53 in LLCC in our study of HBV-related cirrhosis (range: 1.15%-24.06%) is discordant with previous data which have shown low (3%) or absent expression in LLCC; however, the etiology was not taken into account in these studies. More extensive studies may be required to further characterize...
Table 3. Results of Immunohistochemical Stains, TUNEL Assay, Telomere Length Assessment and Senescence-Associated β-Galactosidase Study in Cholestatic Livers (Median [Range]; %)

<table>
<thead>
<tr>
<th></th>
<th>NLH in cholestasis</th>
<th>LLCC in cholestasis</th>
<th>NLH vs LLCC in cholestasis</th>
<th>LLCC in HBV vs cholestasis</th>
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<tr>
<td>Cell cycle checkpoint markers</td>
<td></td>
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<tr>
<td>p21-LI</td>
<td>4.02 (0.46–23.16)</td>
<td>6.98 (2.04–25.69)</td>
<td>n.s.</td>
<td>–</td>
</tr>
<tr>
<td>p27-LI</td>
<td>3.27 (1.14–20.29)</td>
<td>7.41 (0.84–20.96)</td>
<td>n.s.</td>
<td>–</td>
</tr>
<tr>
<td>p16-LI</td>
<td>1.13 (0.00–3.87)</td>
<td>2.53 (0.00–5.56)</td>
<td>P = 0.021</td>
<td>–</td>
</tr>
<tr>
<td>Tp53-LI</td>
<td>0</td>
<td>0</td>
<td>n.s.</td>
<td>▲: P &lt; 0.001</td>
</tr>
<tr>
<td>DNA damage and chromosomal instability</td>
<td></td>
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<td></td>
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<tr>
<td>γH2AX-LI</td>
<td>2.24 (0.00–4.87)</td>
<td>1.27 (0.00–7.83)</td>
<td>n.s.</td>
<td>▲: P &lt; 0.001</td>
</tr>
<tr>
<td>Micronuclei</td>
<td>0</td>
<td>0</td>
<td>n.s.</td>
<td>▲: P &lt; 0.001</td>
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<tr>
<td>Proliferation and apoptosis</td>
<td></td>
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<tr>
<td>PCNA-LI</td>
<td>1.30 (0.00–6.01)</td>
<td>0.65 (0.00–5.39)</td>
<td>n.s.</td>
<td>▲: P &lt; 0.001</td>
</tr>
<tr>
<td>Ki-67-LI</td>
<td>3.27 (0.83–13.62)</td>
<td>1.43 (0.00–6.67)</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>TUNEL-LI</td>
<td>1.23 (0.11–2.18)</td>
<td>1.92 (0.00–4.27)</td>
<td>n.s.</td>
<td>▼: P &lt; 0.001</td>
</tr>
<tr>
<td>M30-LI</td>
<td>0.00 (0.00–0.88)</td>
<td>0.00 (0.00–2.63)</td>
<td>n.s.</td>
<td>▼: P = 0.033</td>
</tr>
<tr>
<td>PCNA-LI - TUNEL-LI</td>
<td>0.33 (–1.87–3.99)</td>
<td>–1.15 (–3.45–4.24)</td>
<td>n.s.</td>
<td>▲: P &lt; 0.001</td>
</tr>
<tr>
<td>Ki-67-LI - TUNEL-LI</td>
<td>1.93 (–0.40–12.00)</td>
<td>0.97 (–4.27–4.24)</td>
<td>P = 0.029</td>
<td>n.s.</td>
</tr>
<tr>
<td>PCNA-LI - M30-LI</td>
<td>1.30 (–0.57–5.44)</td>
<td>0.54 (–2.63–4.28)</td>
<td>n.s.</td>
<td>▲: P &lt; 0.001</td>
</tr>
<tr>
<td>Ki-67-LI - M30-LI</td>
<td>2.57 (0.83–13.62)</td>
<td>1.18 (–2.63–6.67)</td>
<td>P = 0.021</td>
<td>n.s.</td>
</tr>
<tr>
<td>Telomere length</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TFI/CFI ratio</td>
<td>1.474 (0.937–1.849)</td>
<td>1.423 (0.688–1.741)</td>
<td>n.s.</td>
<td>▼: P &lt; 0.001</td>
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<tr>
<td>Senescence marker</td>
<td></td>
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<tr>
<td>SA-β-Gal</td>
<td>5.81 (0.00–49.80)</td>
<td>8.54 (0.00–48.21)</td>
<td>n.s.</td>
<td>▼: P = 0.017</td>
</tr>
</tbody>
</table>

CFI, centromere fluorescent intensity; HBV, hepatitis B-virus; LI, labeling index; LLCC, large liver cell change; NLH, normal-looking hepatocyte; n.s., not statistically significant; TFI, telomere fluorescent intensity.

▲: Higher in HBV-related LLCC than in cholestasis-related LLCC.
▼: Lower in HBV-related LLCC than in cholestasis-related LLCC.

Fig. 8. Comparison of (A) p16-LI, (B) γH2AX-LI, (C) net cellular gain (PCNA-LI – M30-LI), and (D) TFI/CFI ratio between HBV-related cirrhosis and cholestatic livers. Normal-looking hepatocytes are marked by empty boxes and LLCC are marked by shaded boxes.
the Tp53 overexpression status of LLCC, and whether p53 expression is related to p53-HBx inactivation in HBV-infected hepatocytes also remains to be determined.

The loss of cell cycle checkpoint markers has been suggested to allow clonal expansion of hepatocytes with dysfunctional, shortened telomeres. Similarly, in this study of HBV-related hepatocarcinogenesis, statistically significant telomere shortening was seen between normal hepatocytes and NLCH (P = 0.010), and between NLCH and LLCC (P = 0.001). In addition, γ-H2AX foci and micronuclei indices were extremely low in normal hepatocytes with a significant gradual increase from NLCH, LLCC, SLCC, to HCC, reflecting the increasing degree of DNA damage and chromosomal instability associated with loss of cell cycle checkpoints and dysfunctional telomeres. However, although there was a general decrease in telomere length from LLCC, SLCC, to HCC, the differences in telomere lengths were not statistically significant. The limited number of cases enrolled in this study may account for the lack of statistical significance between the three lesions.

It has been suggested that LLCC may represent a “dead-end” of hepatocarcinogenesis. Morphologically, it seems less likely that there is a transition between LLCC and SLCC/HCC. LLCC might actually represent cellular senescence in the context of a tumor suppressor mechanism preventing the proliferation of genetically unstable precancerous cells. Alternatively, oncogene-induced senescence, defined as the activation of oncogenes which induce a senescence checkpoint, might be a possible mechanism for LLCC formation. Both hypotheses suggest that LLCC is a defensive mechanism against hepatocarcinogenesis. The SA-β-Gal activity was weaker and less frequent in LLCC compared to NLCH, which would be unexpected if LLCC represented a population of terminally differentiated end-stage hepatocytes. Interestingly, the overall increase in net cellular gain (high proliferative activity, low apoptotic index) from normal hepatocytes, NLCH, LLCC, SLCC, and HCC in this study, similar to results of a previous report by Koo et al., indicates that LLCC, at least in HBV-related cirrhosis, is not a senescent lesion.

Contradictory results have been previously reported by Lee et al., showing a low proliferative rate and greater degree of apoptosis in their cells with LLCC compared to normal hepatocytes. LLCC has been reported to be observed in various liver diseases such as autoimmune hepatitis, alcoholic cirrhosis, and cholestatic liver, although it is more prevalent in HBV-associated chronic liver disease; therefore, it may be possible that the nature of LLCC is heterogeneous, depending on the biological setting where it arises. Actually in this study, LLCC that was associated with chronic HBV infection demonstrated molecular characteristics different from that in chronic cholestasis. In chronic cholestasis, the telomere lengths, DNA damage, and chromosomal instability (γ-H2AX-LI, micronuclei index), senescence marker (SA-β-Gal activity), and cell dynamics in LLCC were not significantly different from adjacent normal-looking hepatocytes. Tp53 was not expressed in both LLCC and normal-looking hepatocytes in cholestasis, whereas an increase in Tp53-LI was seen in HBV-related LLCC. As for cell cycle checkpoint markers, p16 was significantly increased in cholestatic LLCC compared to normal-looking hepatocytes, and p21-LI and p27-LI were similar both in normal-looking livers and LLCC of chronic cholestasis. This is in contrast to HBV-related LLCC, where the cell cycle checkpoint markers were inactivated compared to NLCH. These findings imply that LLCC arising in cholestatic livers may represent reactive change with a more stringent cell cycle checkpoint control, as previously suggested. Concerning the cell cycle checkpoint markers, we have not directly compared the data between cholestatic and HBV-related LLCC, because they start from two different populations; cholestatic LLCC arises from the noncirrhotic hepatocyte and HBV-related LLCC arises from the cirrhotic hepatocyte where cell cycle checkpoint markers (p21, p27, and p16) are activated.

Several prospective and retrospective studies have shown the presence of LLCC in needle biopsies of HBV-related chronic liver disease is an important independent risk factor for hepatocarcinogenesis, and together with the results of this study, it may be suggested that LLCC in HBV-related chronic liver disease is not simply an innocent reactive lesion, but a dysplastic lesion closely related to hepatocarcinogenesis.

References


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