Abstract

Mallory bodies (MBs) and hyaline globules (HG) are recognized as hepatocellular cytoplasmic inclusions in liver diseases. We reviewed 123 intrahepatic cholangiocarcinomas (ICCs) and encountered 16 cases (13.0%) in which cancer cells had MB-type inclusions and/or HG-type inclusions, both of which are positive for p62 and ubiquitin. The HG type was present in all 16 cases, and 5 cases contained the MB type. Of 16 patients, 12 had chronic liver disease that was related to alcoholic abuse in 4, hepatitis B surface antigen-positive in 3, and hepatitis C virus antibody-positive in 8. Viral infection and liver cirrhosis were more common in ICCs with p62+ inclusions ($P = .0004$ and $P = .0199$, respectively). Of 16 ICCs, 15 with hyaline inclusions had a peripheral tumor location ($P = .0052$). On ultrastructural examination, the MB type had an electron-dense fibrillar appearance, while the HG type appeared as rounded masses of granular materials. Our results suggest that intracytoplasmic hyaline bodies occasionally can be found in cholangiocarcinoma with chronic liver disease related to viral hepatitis or alcoholic intake.

Mallory bodies (MBs) and hyaline globules (HG) are characteristic hepatocellular cytoplasmic inclusions in the liver.$^{1,2}$ The MB is a form of hyaline degeneration of hepatocytes seen in hepatocellular carcinoma (HCC) and liver diseases, including alcoholic hepatitis, nonalcoholic steatohepatitis, and cholestatic liver diseases, such as primary biliary cirrhosis.$^{1-5}$ Intracytoplasmic HGs have been well recognized as a histologic finding in HCCs,$^{6-10}$ but HGs and MBs can be found in pheochromocytoma, renal cell carcinoma, and breast and lung cancers.$^{11-14}$

p62 is a stress-inducible protein that has a role as an adapter molecule in cytokine signaling pathways.$^{15,16}$ It is also a major component of MBs and HGs present in tumor cytoplasm of HCCs.$^{4,17}$ MBs consist of aggregated keratins, particularly keratin 8, ubiquitin, heat shock proteins, and p62.$^{18-21}$ However, HGs lack keratin and differ from MBs in their morphologic appearance.$^{2,22}$ In contrast with the irregularly shaped MBs, HGs are well-circumscribed, homogeneous, eosinophilic globules.$^{2,6}$ p62 was isolated by enzyme-linked immunosorbent assay in HCC$^{23};$ however, 1 of 2 cases of cholangiocarcinoma expressed p62 in cancer cells.$^{24}$ In addition, p62 expression is observed in carcinomas of the gastrointestinal tract and in aggressive breast cancers.$^{25-27}$

It remains unknown whether intrahepatic cholangiocarcinoma (ICC) with p62+ hyaline inclusions exhibits any specific or characteristic clinicopathologic features. We reviewed a series of 123 patients with ICCs and encountered 16 cases in which cholangiocarcinoma cells showed cytoplasmic changes resembling MBs and HGs. Based on the histologic, immunohistochemical, and ultrastructural observations, we clarified the clinicopathologic characteristics of cholangiocarcinoma with hyaline inclusions and...
investigated whether the nature of the inclusions was the same as or different from that of HCC.

Materials and Methods

Tissue Specimens and Patients

We reviewed the sections of 123 surgically resected cases of ICCs submitted to the Department of Anatomic Pathology, Kyushu University, Fukuoka, Japan, from 1985 to 2007. The histopathologic definition of ICC was based on the classification proposed by the World Health Organization. In this study, cholangiocarcinoma cases included the definite adenocarcinoma component arranged in a tubular manner or nests with mucin production. The HCC-like trabecular pattern was not included. All 123 cases were positive for cytokeratin (CK) and/or MOC-31 by immunohistochemical analysis. Cases with other primary adenocarcinomas, such as of the gastrointestinal tract, breast, or lung, were excluded. Mucin production of cancer cells was confirmed by staining with alcian blue and periodic acid–Schiff (PAS) with diastase. Whenever possible, multiple sections of tumor were examined, and we obtained tumors with intracytoplasmic hyaline inclusions, which contained MB-type and HG-type inclusions. We defined MB-type inclusions as irregular reticular eosinophilic inclusions identical to alcoholic hyaline and HG-type inclusions as sharply circumscribed, round or oval, eosinophilic globular bodies partly surrounded by clear halos. Cases of cholangiocarcinoma with an area of Hep Par 1–positive or α-fetoprotein–positive carcinoma cells, indicating combined HCC and cholangiocarcinoma, and cholangiocarcinoma with rhabdoid features were excluded. ICCs lacking hyaline inclusions were studied for comparison.
The patients included in the study ranged in age from 33 to 90 years (mean, 63.9 years); 78 were men and 45 were women. Hepatitis B surface antigen was positive in 16 cases, and hepatitis C virus (HCV) antibody was positive in 23 cases. Liver cirrhosis was found in 21 cases. The mean tumor diameter was 4.23 cm (range, 1-12 cm), and vascular invasion was positive in 79 cases (64.2%). Tumor location was divided into a hilar type of ICC (n = 51), which involved a large bile duct, and a peripheral type of ICC (n = 72), which involved bile duct smaller than segmental branches, based on the gross and histologic classification in a previous study. Sections of the nontumorous liver were examined for the presence of cirrhosis and the presence of MBs in adjacent hepatocytes.

Our study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. For strict privacy protection, identifying information for all samples was removed before analysis.

**Immunohistochemical Analysis**

Formalin-fixed specimens were embedded in paraffin. De-paraffinized and rehydrated 4-μm sections were stained with H&E for microscopic evaluation. In addition, PAS staining with or without diastase for hyaline inclusions was performed.

For immunohistochemical studies, we selected representative specimens, and the following primary antibodies were used: p62 (guinea pig polyclonal, dilution 1:200; Progen, Heidelberg, Germany), CK19 (mouse monoclonal, dilution 1:100; DAKO, Glostrup, Denmark), CK8 (mouse monoclonal, dilution 1:100; DAKO, Carpinteria, CA), MOC-31 (mouse monoclonal, dilution 1:200; DAKO, Carpinteria), ubiquitin (rabbit polyclonal, dilution 1:500; DAKO, Glostrup), and vimentin (mouse monoclonal, dilution 1:25; DakoCytomation, Carpinteria). These primary antibodies were incubated at 4°C overnight. The subsequent reaction was carried out using a streptavidin-biotin-peroxidase method (Histofine, Nichirei, Tokyo, Japan). Peroxidase reactivity was visualized by using 3,3’-diaminobenzidine. No significant staining was observed in the negative control samples, which were prepared using the mouse immunoglobulin at the same concentration. For positive control samples, hepatocyte for CK8, bile duct for CK19 and MOC-31, muscular cells for vimentin, and neural inclusions of Alzheimer disease for p62 and ubiquitin were used. MBs consist of p62 protein and keratins, particularly CK8, whereas HGs lack keratins, so we performed double immunostaining with p62 and CK8. CK8 staining was developed with a peroxidase-conjugated secondary antibody, and p62 staining was developed with an alkaline phosphatase–conjugated secondary antibody.

**Electron Microscopy**

Electron microscopic examination was performed in 4 cases using standard methods on tissues reprocessed from the paraffin blocks.

**Statistical Analysis**

Statistical analysis to compare the relationships between the presence of hyaline inclusions and clinicopathologic factors was performed by using the χ² test, Fisher exact test, and Student t test for tumor size. Patient survival was defined as the period of survival between surgery and the date of the last follow-up or until death of disease. Survival curves were compared by using the log-rank test. A P value of less than .05 was considered statistically significant.

**Results**

**Hyaline Inclusions and Immunohistochemical Results**

The light microscopic examination of 123 cholangiocarcinomas showed that hyaline inclusions were present in 16 cases (13.0%). Hyaline inclusions were not homogeneously distributed and were commonly located in the cytoplasm of cancer cells. Table I summarizes the clinicopathologic characteristics and immunohistochemical results for 16 patients with ICC with hyaline inclusions. The mean age of the patients was 66.0 years (range, 50-88 years). Of the 16 cases, 10 were in men and 6 in women. HG-type inclusions were present in all 16 cases and 5 cases contained MB-type inclusions. PAS stain with or without diastase was negative for MB-type and most HG-type inclusions. Most hyaline inclusions in all 16 cases were strongly positive for p62 and vimentin expression was in contrast, 107 ICCs without hyaline inclusions were negative for p62. One case with the HG type showed no CK8 expression and CK8 was expressed in many MBs and in a small number of HGs in 5 cases with both MBs and HGs. Ubiquitin was also expressed in all 16 cases with hyaline inclusions and was detected in many but not all hyaline inclusions. Vimentin expression was not observed in any MBs.

A review of medical records revealed that 4 patients had a history of alcohol abuse, 3 patients were positive for hepatitis B surface antigen, and 8 patients were positive for HCV antibody. Four patients had no viral infection and no history of alcohol abuse. Liver cirrhosis was present in 6 cases, and 4 alcoholic livers revealed MBs of the hepatocytes in nontumorous tissue.

**Electron Microscopic Findings**

On ultrastructural examination in 4 cases, tumor cells having MB-type inclusions showed an electron-dense, fibrillar, or...
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The evaluation of intracytoplasmic hyaline inclusions of cho-

Table 1
Clinicopathologic Findings in 16 Patients With Intrahepatic Cholangiocarcinomas With p62+ Hyaline Inclusions

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex/Age (y)</th>
<th>Hyaline Type</th>
<th>PAS</th>
<th>CK8</th>
<th>Ubiquitin</th>
<th>Viral Infection/Alcohol Abuse</th>
<th>Liver Cirrhosis</th>
<th>Mallory Bodies in Nontumor Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/58</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/F/50</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/F/57</td>
<td>HG and MB</td>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>HCVAb+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>4/F/74</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>5/M/74</td>
<td>HG and MB</td>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>HCVAb+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>6/F/73</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>HBsAg+/alcohol</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7/F/64</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>HCVAb+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>8/M/66</td>
<td>HG</td>
<td></td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>HCVAb+/alcohol</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>9/M/63</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>Alcohol</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10/M/80</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>HCVAb+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>11/M/63</td>
<td>HG and MB</td>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>HCVAb+/alcohol</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12/M/50</td>
<td>HG and MB</td>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>HBsAg+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>13/M/77</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>HCVAb+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>14/F/88</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>15/M/57</td>
<td>HG and MB</td>
<td></td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>HCVAb+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>16/M/62</td>
<td>HG</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>HBsAg+</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Ab, antibody; CK, cytokeratin; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HG, hyaline globule; MB, Mallory body; PAS, periodic acid-Schiff; +, positive or present; –, negative or absent.

* Diastase PAS+.

A granular amorphous appearance Image 4A, whereas tumor cells of HG-type inclusions showed a rounded matrix consisting of electron-dense, granular materials with lighter vacuoles Image 4B and Image 4C.

Comparison With Control Cases

The comparison of the presence of p62+ hyaline inclusions in ICCs and clinicopathologic factors is summarized in Table 2. Viral infection with HBV or HCV and liver cirrhosis were more common in ICCs with p62+ hyaline inclusions than in ICCs without (P = .0004 and P = .0199, respectively). All ICCs having hyaline inclusions except for 1 case had a peripheral tumor location (P = .0052). Significant differences were not detected between the presence of hyaline inclusions of ICCs and other clinicopathologic factors. The survival rates for patients with p62+ ICC at 3 and 5 years were 50.3% and 25.2%, respectively, whereas those for patients with p62–ICC at 3 and 5 years were 47.2% and 37.8%, respectively. There was no statistical difference in survival rates between the 2 groups.

Discussion

Hyaline inclusions are characteristic features of HCC, but malignant liver tumors with rhabdoid features and hepatic embryonal sarcoma have a globular hyaline-like structure. The evaluation of intracytoplasmic hyaline inclusions of cholangiocarcinoma is extremely limited, and hyaline inclusions have not been reported in cholangiocarcinoma. However, on the basis of histologic and immunohistochemical observations, 16 (13.0%) of 123 cases of human ICC were found to contain hyaline inclusions, which are reactive for p62 and ubiquitin proteins. The ICCs with hyaline inclusions that we examined contained no definite HCC areas.

In our study, 2 types of hyaline inclusions were recognized: the MB type, which was morphologically identical to the type described by Mallory as alcoholic hyaline, and the HG type, which was made up of sharply circumscribed, oval or round masses. Norkin and Campagna-Pinto demonstrated that reticular hyaline identical to MBs was never stained by PAS stain; however, the PAS stain of HGs was variable and controversial.6,9,10,32 Our identified MBs were negative for PAS, and a small number of HGs were positive for PAS. If abnormal keratins are present in addition to p62, leading to MBs, and if p62 is induced alone, HGs may arise, and morphologic transitions of HGs and MGs have been reported.17,33 In the present study, 5 cases showed mixed MB and HG types, an observation that might have resulted from a coincidental association of both types of inclusion. Hyaline inclusions of ICCs as determined by electron microscopic study show striking similarities to those of HCCs. Hyaline inclusions of HCCs include MBs, megamitochondria, lysosome, and endoplasmic reticulum.7,9,10,33,34 MB-type inclusions of ICC consist of an electron-dense granular appearance, presumably alcoholic hyaline, which is regarded as PAS– in HCCs,9 while some HG-type inclusions of ICC appear as masses of granular materials with lighter vacuoles identical to the giant lysosomes detected in HCCs.7,10

Liver cirrhosis, chronic viral hepatitis including HBV and HCV infection, and heavy alcohol consumption have been recognized as risk factors for the development...
A and B. Well-differentiated adenocarcinoma with hyaline globule (HG)-type inclusion (A, H&E, ×400; B, H&E, ×800). C. The HG type was negative for periodic acid–Schiff stain (arrows; ×800). D. The HG type was extensively positive for p62 (×800). E. Double staining for p62/cytokeratin (CK8) revealed that HG-type inclusions were positive for p62 (red), but negative for CK8; a nonneoplastic small bile duct as an endogenous control was positive for CK8 (brown) (×800). F. HG-type inclusions were negative for vimentin (×800).
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**Image 3** A and B. Adenocarcinoma with hyaline inclusions lacking clear glandular structure in which mucin production was confirmed by alcian blue staining (A, H&E, ×400; B, alcian blue, ×400). C. Varied sizes of Mallory body (MB)-type inclusions were seen in the cancer cytoplasm (higher-power view of A; ×800). D. MB-type inclusions were negative for periodic acid-Schiff (PAS) (arrows), and a few hyaline globule (HG)-type inclusions were positive with PAS with diastase (arrowhead) (×800). MB-type inclusions were positive for p62 (E, ×800), cytokeratin 8 (F, ×400), and ubiquitin (G, ×800). A few HG-type inclusions were positive for cytokeratin 8 (arrow) (F).
of cholangiocarcinoma.\textsuperscript{35-40} Yamada et al\textsuperscript{41} suggested that interlobular bile duct damage observed in alcoholic injury was similar to that of viral hepatitis. Bile duct dysplasia can be found in the pathologic conditions of HCV infection and alcohol intake.\textsuperscript{42} An important mechanism implicated in alcohol-related and HCV-associated hepatocarcinogenesis is oxidative stress.\textsuperscript{43,44} DNA fragmentation indicating the generation of reactive oxygen reflects a genotoxic effect of alcohol or HBV and HCV in hepatocarcinogenesis.\textsuperscript{45} Aggregation of p62 and misfolded keratin, major components of hyaline inclusions, were preferentially induced by chronic oxidative stress.\textsuperscript{46-49} In this study, ICCs with hyaline inclusions showing peripheral tumor location were related to viral infection and liver cirrhosis. Therefore, viral infection, such as HBV or HCV, and alcohol intake may contribute to the formation of hyaline inclusions and cholangiocarcinogenesis in peripheral-type ICC. Combined HCC and cholangiocarcinoma, mixed phenotype, seems to be arising from a common bipotential progenitor cell.

**Table 2**  
Comparison of Intrahepatic Cholangiocarcinomas With or Without p62+ Hyaline Inclusions  

<table>
<thead>
<tr>
<th></th>
<th>With Hyaline Inclusions (n = 16)</th>
<th>Without Hyaline Inclusions (n = 107)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>10/6</td>
<td>68/39</td>
<td>.9351</td>
</tr>
<tr>
<td>Viral infection present</td>
<td>11</td>
<td>24</td>
<td>.0004</td>
</tr>
<tr>
<td>Liver cirrhosis present</td>
<td>6</td>
<td>15</td>
<td>.0199</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilar/peripheral</td>
<td>1/15</td>
<td>50/57</td>
<td>.0052</td>
</tr>
<tr>
<td>Mean ( \pm ) SD tumor size (cm)</td>
<td>4.1 ( \pm ) 1.7</td>
<td>4.3 ( \pm ) 2.2</td>
<td>.7567</td>
</tr>
<tr>
<td>Histologic differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well and moderately/poorly</td>
<td>10/6</td>
<td>77/30</td>
<td>.6286</td>
</tr>
<tr>
<td>Vascular invasion present</td>
<td>13</td>
<td>66</td>
<td>.2129</td>
</tr>
<tr>
<td>Survival after surgery (%)</td>
<td></td>
<td></td>
<td>.9819</td>
</tr>
<tr>
<td>3-γ</td>
<td>50.3</td>
<td>47.2</td>
<td></td>
</tr>
<tr>
<td>5-γ</td>
<td>25.2</td>
<td>37.8</td>
<td></td>
</tr>
</tbody>
</table>

\* Data are given as number of cases unless otherwise indicated.
Hyaline inclusions were commonly found in HCC cells; therefore, some cholangiocarcinomas having hyaline inclusions may be derived from a bipotential progenitor cell.

Immunohistochemical stains for p62, ubiquitin, and CK8, as well as electron microscopy, reveal that hyaline inclusions detected in ICCs resemble those confirmed in HCCs. Although the presence of intracellular hyaline inclusions in liver tumors as obtained by core needle biopsy or aspiration cytology is supportive of a diagnosis of HCC, our results suggest that intracytoplasmic hyaline inclusions occasionally can be found in cholangiocarcinoma with chronic liver disease related to viral hepatitis or alcohol intake.

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