Differential Diagnostic Value of GPC3-CD34 Combined Staining in Small Liver Nodules With Diameter Less Than 3 cm

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Abstract

The diagnostic value of combining glypican-3 (GPC3) and CD34 staining for small nodules in liver biopsy specimens has not been evaluated. In this study, 201 thin-core biopsy specimens were assessed using GPC3 and CD34 immunochemical staining, including 33 cirrhotic regenerative nodules, 31 high-grade dysplastic nodules, 70 hepatocellular carcinomas (HCCs) with nodules 3 cm or smaller, and 67 HCCs with nodules larger than 3 cm. The results showed that the accuracy of GPC3 staining (90.3%) among liver nodules 3 cm or smaller was better than its use among all nodules (P = .045). Furthermore, the positive expression rate of costaining was significantly greater than that observed for GPC3 or CD34 single staining (P < .001 and P = .002, respectively). These data demonstrate that GPC3 staining is more accurate for the diagnosis of HCC on thin-core biopsy specimens in nodules 3 cm or smaller compared with its use in all nodules, while GPC3 and CD34 costaining has better diagnostic value than does single staining.

Hepatocellular carcinoma (HCC) is one of the most malignant human cancers and affects a large population worldwide. According to a report in 2002, China has one of the highest rates of HCC prevalence throughout the world. Among 620,000 new incident cases every year, 55% of them are from China. Many people with HCC do not have symptoms until tumor growth has reached intermediate or advanced stages, so the early diagnosis of HCC is very important for successful treatment.

Recently, with the development of new radiologic diagnostic techniques, even small hepatic nodules can be detected. However, many nodules may lack the characteristic appearance or typical vascular profile, and liver biopsies need to be performed to make accurate diagnoses. Sangiovanni et al found that biopsy is required for the final diagnosis in approximately 55% of patients for whom only 1 radiologic examination was conducted. Leoni et al reported that although 3 radiologic methods were used to exclude the possibility of HCC, approximately 20% of lesions were still confirmed by pathologic examinations to be malignant. However, the morphologic features that distinguish high-grade dysplastic nodules (HGDNs) from HCC, namely stromal invasion, may not be easily detected in biopsy samples through conventional histologic staining. Therefore, it is helpful to stain several HCC markers to differentiate malignant from benign tissue.

Glypican-3 (GPC3) is a member of the glypican family, a group of heparan sulfate proteoglycans linked to the cell surface through a glycosyl-phosphatidylinositol anchor. The mutation of GPC3 can lead to an imbalance in cell
proliferation and apoptosis and may be an important cause of tumorigenesis. Several studies have shown increased GPC3 levels in serum, resected tissues, and liver biopsy specimens from patients with HCC. The 2005 American Association for the Study of Liver Diseases (AASLD) guidelines for the management of HCC suggest including GPC3, HSP70, and glutamine synthetase staining to assist in the pathologic diagnosis.

When solid tumors grow to 2 mm in diameter, the further growth of tumor tissue depends on angiogenesis. These newly formed blood vessels penetrate into cancerous tissue, supplying nutrients and oxygen and removing waste products. Because HCC is a hypervascular tumor, staining for vascular endothelium in HCC with CD34 is typically positive or strongly positive as unpaired arteries are more clearly identified, whereas in benign tissue, the sinusoidal epithelium stains only weakly with CD34 antibody. Because CD34 single staining is not very specific for HCC, it was reported that the combination of GPC3 and CD34 staining could improve the differential diagnosis between HGDNs and HCC. However, this study was conducted using resected liver tissues, so whether the use of needle biopsy specimens could be adapted to this purpose remains to be investigated.

As the 2010 Barcelona Clinic Liver Cancer (BCLC) update mentioned, diagnosing liver nodules 3 cm or smaller as early as possible is crucial for achieving higher 5-year survival rates. In the study, we conducted GPC3 and CD34 staining in 134 liver biopsy samples from liver nodules 3 cm or smaller, including 33 pathologically confirmed cirrhotic large regenerative nodules (CLRNs), 31 HGDNs, and 67 HCC cases. The data were analyzed, and the potential of GPC3 and CD34 staining in the differential diagnosis of thin-core biopsy specimens of malignant and benign small liver nodules is discussed.

Materials and Methods

Patients and Samples

This study was approved by the ethics committee of Tianjin Third Hospital, Tianjin, China, and written informed consent was obtained from each participant. In total, 225 liver biopsy specimens were obtained during HCC surveillance in patients with hepatitis B virus–associated liver cirrhosis from January 1, 2006, to June 30, 2009, including 33 pathologically confirmed CLRN cases, 31 HGDN cases, 70 HCC cases with nodules 3 cm or smaller, and 67 HCC cases with nodules larger than 3 cm. The size of the liver nodules was determined through radiologic study or surgical resection. Liver thin-core biopsy specimens were obtained using a 20-gauge sample needle under ultrasound guidance before carrying out any treatment. Patients with chronic hepatitis C, alcoholic hepatitis, or autoimmune hepatitis and patients with Child-Pugh C liver function and pathologically confirmed cholangiocarcinoma were excluded from this study.

Pathologic Study

All samples were fixed with neutral 4% formaldehyde solution. Then 4-μm continuous sections were obtained for H&E staining, reticular fiber staining, iron staining, and Masson staining to make the pathologic diagnosis. GPC3 (clone 1G-12, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and CD34 (clone QBEND 10, dilution 1:50; DAKO, Carpinteria, CA) immunohistologic staining was also conducted. Previously validated slides were used as positive control samples. Slides using phosphate-buffered saline instead of primary monoclonal antibody were regarded as negative control samples. The slides were deparaffinized and rehydrated. Endogenous peroxide was blocked by 3% hydrogen peroxide treatment, and the antigen was retrieved using sodium citrate treatment in a pressure cooker at 120°C for 10 minutes. Bovine serum was used to block slides, and primary antibodies were incubated with slides overnight at 4°C. Finally, biotin-conjugated secondary antimouse IgG antibodies (Zhongshan Biotech, Beijing, China) were added. Immunohistochemical staining was performed according to the manufacturer’s instructions.

Clinical Evaluation

According to the AASLD guidelines for the management of HCC, serum α-fetoprotein level, abdominal ultrasound examination, and enhanced computed tomography or magnetic resonance imaging were used to diagnose HCC. Liver cirrhosis was diagnosed based on histologic, serologic, and radiologic test results. Patients with HGDNs were followed up for more than 12 months, and no malignancy was found.

Pathologic Evaluation

The pathologic features of all liver biopsy specimens were evaluated by 2 senior pathologists who were blinded to the clinical information of patients. If permitted, paired liver biopsy and resected tissue samples from the same patients were compared to confirm the diagnosis. The criteria for HCC and HGDN diagnosis were referenced from the World Health Organization and International Consensus Group for Hepatocellular Neoplasia guidelines, and HCC grading was divided into well-differentiated (G1) and moderately to poorly differentiated (G2/G3). According to a previous study, membranous and cytoplasmic GPC3 staining was regarded as positive staining, and the number of immunoreactive cells was quantified. Sections were evaluated as GPC3+ when more than 5% of tumor cells exhibited GPC3+ staining. The label + represented 5% to 10%
immunoreactive cells (low expression), 2+ represented 11% to 50% immunoreactive cells (intermediate expression), and 3+ represented more than 50% immunoreactive cells (high expression). Also according to a previous study, CD34 immunoreactivity was evaluated according to the presence of brownish yellow staining of the HCC microvascular endothelium. Negative immunoreactivity of CD34 was defined as exclusive staining of the vascular endothelium in the portal area and no staining of the adjacent liver sinus endothelium. Complete CD34 immunoreactivity was defined as massive staining of the liver sinus endothelium, and incomplete CD34 immunoreactivity was defined as partial staining of the liver sinus endothelium (including staining of the adjacent sinus endothelium in the portal area).

Statistical Analysis

SPSS, version 12.0 (SPSS, Chicago, IL), was used for statistical analysis. The positive staining percentage of all HCC biopsy specimens represented the sensitivity, and the negative staining percentage of all non-HCC biopsy specimens represented the specificity. Positive predictive value (PPV) equaled the percentage of positive HCCs staining among all positively staining samples. Negative predictive value (NPV) equaled the percentage of negative HCC staining among all negatively staining samples. The accuracy was represented by the proportion of biopsy samples that were correctly diagnosed. The paired \( \chi^2 \) test and Fisher exact test were used to compare among groups. A \( P \) value less than .05 was considered statistically significant.

Results

The clinical parameters for patients enrolled in this study are listed in Table 1. All patients had a history of hepatitis B virus infection and were hepatitis B surface antigen positive. Patients from the CLRN, HGDN, and HCC groups were similar in terms of age, sex, alanine aminotransferase level, total bilirubin level, Child-Pugh score, and serum \( \alpha \)-fetoprotein level. Cases with small HCC nodules were all classified as BCLC early stage, while cases with nodules larger than 3 cm were classified as BCLC intermediate or advanced stage. The statistical analysis suggested that GPC3 or CD34 staining was irrelevant to disease status (\( P > .05 \)).

Glypican-3

GPC3 expression was found in most HCC samples (110/137, 80.3%) as shown in Table 2. No GPC3 expression was found in HGDN and CLRN cases, except that 1 (3%) of 31 HGDN cases showed a low level of GPC3 expression. GPC3 in HCC was found in the cytoplasm or cell membrane, with a diffuse or granule-like distribution. GPC3 expression and the immunoreactive cells score were found to be irrelevant to HCC pathological differentiation (G1 vs G2-G3, \( P > .05 \); Table 2). The representative GPC3 expression patterns from each differentiated HCC group are shown in Image 1. The sensitivity, specificity, PPV, NPV, and accuracy in HCC diagnosis using GPC3 staining were 80.3%, 98%, 99.1%, 84%, and 86.1%, respectively, as shown in Table 3.

CD34

As shown in Image 2, CD34 expression was labeled as negative, incompletely positive, or completely positive. In CD34 completely positive biopsy specimens, most hepatic sinus lesions contained brown deposits; in incompletely positive biopsy specimens, some peripheral and near portal area sinuses were stained brown. All CLRNs were negative. Among HGDNs, 6 (19%) of 31 were incompletely positive, and only 1 (3%) of 31 was completely positive, whereas among HCC nodules, 119 (86.9%) of 137 were completely positive.

Table 1

Clinical Characteristics of Enrolled Patients

<table>
<thead>
<tr>
<th></th>
<th>CLRN (n = 33)</th>
<th>HGDN (n = 31)</th>
<th>HCC ≤3 cm (n = 70)</th>
<th>HCC &gt;3 cm (n = 67)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD age (y)</td>
<td>51 ± 11</td>
<td>52 ± 10</td>
<td>55 ± 11</td>
<td>56 ± 10</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>25/8</td>
<td>23/8</td>
<td>57/13</td>
<td>55/12</td>
</tr>
<tr>
<td>Cirrhosis (Y/N)</td>
<td>33/0</td>
<td>29/2</td>
<td>60/10</td>
<td>62/5</td>
</tr>
<tr>
<td>ALT, ≤40/&gt;40 U/L (≤0.67/0.67 μkat/L)</td>
<td>18/15</td>
<td>17/14</td>
<td>39/31</td>
<td>36/31</td>
</tr>
<tr>
<td>TBil, ≤1.2/1.2 mg/dL (≤20/&gt;20 μmol/L)</td>
<td>20/13</td>
<td>19/12</td>
<td>47/23</td>
<td>43/24</td>
</tr>
<tr>
<td>Child-Pugh score (A/B)</td>
<td>19/14</td>
<td>19/12</td>
<td>49/21</td>
<td>42/25</td>
</tr>
<tr>
<td>Serum HBsAg (+/−)</td>
<td>33/0</td>
<td>31/0</td>
<td>70/0</td>
<td>67/0</td>
</tr>
<tr>
<td>Serum HBeAg (+/−)</td>
<td>10/23</td>
<td>9/22</td>
<td>11/59</td>
<td>13/54</td>
</tr>
<tr>
<td>AFP, ≤20/&gt;20 ng/mL (≤20/&gt;20 μg/L)</td>
<td>29/4</td>
<td>23/8</td>
<td>39/31</td>
<td>35/32</td>
</tr>
<tr>
<td>BCLC grade (0/A/B/C)</td>
<td>NA</td>
<td>NA</td>
<td>17/53/0/0</td>
<td>0/0/44/23</td>
</tr>
<tr>
<td>Pathologic differentiation grade, G1/G2-G3</td>
<td>NA</td>
<td>NA</td>
<td>54/16</td>
<td>14/53</td>
</tr>
</tbody>
</table>

AFP, \( \alpha \)-fetoprotein; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; CLRN, cirrhotic large regenerative nodules; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HGDN, high-grade dysplastic nodules; NA, not adopted; Tibl, total bilirubin.

### Table 2

**Immunohistologic Staining for Glypican-3 and CD34**

<table>
<thead>
<tr>
<th></th>
<th>Glypican-3</th>
<th>CD34</th>
<th>Costaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>CLRN (n = 33)</td>
<td>33 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HGDN (n = 31)</td>
<td>30 (97)</td>
<td>1 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3 cm (n = 70)</td>
<td>12 (17)</td>
<td>17 (24)</td>
<td>16 (23)</td>
</tr>
<tr>
<td>&gt;3 cm (n = 67)</td>
<td>15 (22)</td>
<td>16 (24)</td>
<td>15 (22)</td>
</tr>
<tr>
<td>Grade 1 (n = 68)</td>
<td>13 (19)</td>
<td>19 (28)</td>
<td>12 (18)</td>
</tr>
<tr>
<td>Grade 2/3 (n = 69)</td>
<td>14 (20)</td>
<td>14 (20)</td>
<td>19 (28)</td>
</tr>
</tbody>
</table>

CLRN, cirrhotic large regenerative nodules; HCC, hepatocellular carcinoma; HGDN, high-grade dysplastic nodules; 1+, 5%-10% immunoreactive cells; 2+, 11%-50% immunoreactive cells; 3+, >50% immunoreactive cells.

* Data are given as number (percentage).

**Image 1** Glypican-3 (GPC3)+ staining pattern in hepatocellular carcinoma (HCC) tissues with varying pathologic differentiation (×400). **A.** Cytoplasmic granular expression in grade 1 HCC tissues. **B.** Diffuse cytoplasmic expression in grade 2 HCC tissues. **C.** Diffuse expression in the cell membrane and cytoplasm in grade 2 HCC tissues. **D.** Diffuse cytoplasmic and membranous expression in grade 3 HCC tissues.
positive, 6 (4.4%) of 137 were incompletely positive, and only 12 (8.8%) of 137 were negative (Table 2). CD34 expression was not related to HCC pathologic differentiation (Table 2). The sensitivity, specificity, PPV, NPV, and accuracy in HCC diagnosis using CD34 staining were 91.2%, 89%, 94.7%, 83%, and 90.5%, respectively, as shown in Table 3.

The statistical analysis suggested that GPC3 and CD34 single staining were significantly different in the HGDN group ($P = .008$), with no difference in small HCC nodules ($\leq 3 \text{ cm}; P = .541$) or larger nodules ($> 3 \text{ cm}; P = .302$).

**GPC3 and CD34 Costaining**

The lack of GPC3 and CD34 staining was regarded as negative staining, while a positive result for either was regarded as positive staining. As shown in Table 2, 128 HCC cases among 137 were positive (93.4%), only 8 HGDN cases

**Table 3**

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glypican-3</td>
<td>110/137 (80.3)</td>
<td>63/64 (98)</td>
<td>110/111 (99.1)</td>
<td>63/75 (84)</td>
<td>86.1</td>
</tr>
<tr>
<td>CD34</td>
<td>125/137 (91.2)</td>
<td>57/64 (89)</td>
<td>125/132 (94.7)</td>
<td>57/69 (83)</td>
<td>90.5</td>
</tr>
<tr>
<td>Costaining</td>
<td>128/137 (93.4)</td>
<td>56/64 (88)</td>
<td>128/136 (94.1)</td>
<td>56/65 (86)</td>
<td>91.5</td>
</tr>
</tbody>
</table>

* Data are given as number/total (percentage) unless otherwise indicated.
among 31 were positive (26%), and all CLRN cases were negative. The sensitivity, specificity, PPV, NPV, and accuracy in HCC diagnosis using co-staining were 93.4%, 88%, 94.1%, 86%, and 91.5%, respectively (Table 3). The characteristic changes in the GPC3 and CD34 co-staining pattern are shown in Image 3. The receiver operating characteristic curve for HCC diagnosis using GPC3 staining, CD34 staining, or co-staining is shown in Image 3. The results revealed that the positivity of co-staining was not different between nodules 3 cm or smaller and nodules larger than 3 cm ($P = .221$); however, the positivity was significantly different between HGDNs and HCC nodules ($P < .001$), indicating that
GPC3 and CD34 costaining could assist in the diagnosis of malignant and benign liver nodules.

Significance of GPC3 and CD34 Costaining in Small HCC Nodules

As shown in Table 2, for patients with small HCC nodules (≤3 cm), the positivity of GPC3 and CD34 was 58 of 70 (83%) and 66 of 70 (94%), respectively, while the positivity of combined staining increased to 67 of 70 (96%), which is significantly higher than that for GPC3 and CD34 single staining (P < .001 and P = .002, respectively). In addition, the sensitivity, specificity, PPV, NPV, and accuracy achieved using GPC3 staining were 83%, 98%, 98%, 84%, and 90.3%, respectively; the sensitivity, specificity, PPV, NPV, and accuracy using CD34 staining were 94%, 89%, 90%, 93.4%, and 91.8%, respectively, while the positivity, sensitivity, specificity, PPV, NPV, and accuracy using costaining were 96%, 88%, 89%, 95%, and 91.8%, respectively. Table 4. The accuracy of GPC3 staining for the diagnosis of HCC among all HCC nodules (Tables 3 and 4; P = .023). A typical CD34 and GPC3 costaining image of small HCC nodules is shown in Image 4.

Discussion

The 2005 AASLD guidelines for the management of HCC suggest that patients with liver nodules that have a non-specific vascular profile should continue to undergo enhanced follow-up or liver biopsy examination. However, the morphologic features that distinguish HGDNs from HCC, namely stromal invasion, may not be detected using conventional H&E staining or reticular fiber staining from a liver biopsy sample, so staining for several histologic markers of HCC may be helpful. The 2010 BCLC update also indicated that patients with early-stage HCC with solitary HCC lesions and preserved liver function (Child-Pugh A and B) with up to 3 HCC nodules of a diameter of 3 cm or less can be effectively treated by resection, liver transplantation, or percutaneous ablation with the possibility of a long-term cure and a high 5-year survival percentage. It is therefore important to obtain an early and accurate diagnosis of HCC among patients with liver nodules of a diameter of 3 cm or less.

Recently, studies of the GPC3, HSP70, and glutamine synthetase staining features in resected HCC tissues and needle biopsy specimens showed that combination staining was more specific in differentiating between HGDN and early-stage HCC nodules. However, the combination of all 3 markers increased the complexity and difficulty of diagnosis, such that simplification of the procedure was needed.

Because HCC is a hypervascular tumor, HCC marker GPC3 and vascular endothelium marker CD34 were studied and evaluated for their diagnostic value in nodules with a diameter of 3 cm or less. We found that the sensitivity and specificity of GPC3 single staining for all HCC nodules were 80.3% and 98%, respectively, while for nodules 3 cm or smaller, the values were 83% and 98%, suggesting that GPC3 staining helps achieve an accurate diagnosis. We also found that GPC3 expression in benign nodules 3 cm or smaller was very low; the positive rate in HGDN was only 3.2%, while there was no expression in CLRN. These results are consistent with previous reports and revealed the value of GPC3 in differentiating between malignant nodules and HGDNs. Moreover, in addition to these studies, we also found GPC3 staining to be more accurate in HCC nodules 3 cm or smaller than its use in all HCC nodules, further revealing its diagnostic value in small HCC lesions.

Although Tátrai et al reported that agrin expression was more specific than CD34 expression in HCC vascular staining, CD34 is still widely used as a vascular endothelium marker. CD34 staining is one of the important markers of tumor angiogenesis and microvascular density in HCC and is closely associated with HCC prognosis. In the current study, using liver thin-core biopsy specimens, we found that the sensitivity and specificity of CD34 expression in small HCC nodules (≤3 cm) were 94% and 89%, respectively. Moreover, 90% of the samples were completely positive, suggesting that the sensitivity of CD34 staining is much higher than that of GPC3 staining (P < .05). No CD34 expression was found in CLRN, and 77.4% of HGDN samples were CD34–. In contrast with the report by Coston et al, although

| Table 4 |
| Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, and Accuracy of Glypican-3, CD34, and Costaining in Liver Lesions 3 cm or Smaller |

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glypican-3</td>
<td>58/70 (83%)</td>
<td>63/64 (98%)</td>
<td>58/59 (98)</td>
<td>63/75 (84)</td>
<td>90.3</td>
</tr>
<tr>
<td>CD34</td>
<td>66/70 (94%)</td>
<td>57/64 (89%)</td>
<td>66/73 (90)</td>
<td>57/61 (93)</td>
<td>91.8</td>
</tr>
<tr>
<td>Costaining</td>
<td>67/70 (96%)</td>
<td>56/64 (88%)</td>
<td>67/75 (89)</td>
<td>56/59 (95)</td>
<td>91.8</td>
</tr>
</tbody>
</table>

* Data are given as number/total (percentage) unless otherwise indicated.
19% of HGDN samples were incompletely CD34+, the expression pattern was different from that observed in HCC, indicating that CD34 staining has diagnostic value in differentiating small hepatic nodules.

By comparing GPC3 and CD34 staining, we found that costaining is much better than single staining for diagnosing small liver nodules (≤3 cm). This result is consistent with the results reported by Coston et al\textsuperscript{18} in which resected liver tissues were used for histologic studies, indicating that GPC3 and CD34 costaining in liver thin-core biopsy specimens can help differentiate small malignant and benign liver nodules and can provide evidence for proper treatment and improve the prognosis for patients with HCC.

For the diagnosis of HCC using liver thin-core biopsy specimens from nodules 3 cm or smaller, GPC3 staining is more specific and accurate in HCC nodules 3 cm or smaller compared with its use in all nodules, and CD34 staining is more sensitive, while GPC3 and CD34 costaining is more valuable than single staining.

Image 4 (Case 2097407) Glypican-3 (GPC3) and CD34 expression in small hepatocellular carcinoma (HCC) sample. Pathologic diagnosis of early-phase HCC in biopsy and resection samples. A, The maximum diameter of the surgically removed tumor was 1.6 cm. B, H&E staining, incompletely positive expression of CD34, and diffuse cytoplasmic expression of GPC3.
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