Utility of an immunostaining panel for diagnosis of hepatocellular carcinoma in fine needle-aspiration biopsies of the liver

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Abstract: Background: Histological identification of liver nodules between hepatocellular carcinoma (HCC) and other lesions can be full of challenges sometimes, especially in fine-needle aspiration (FNA) biopsies. Our aim was to investigate the efficacy of combined use of Arginase-1, Glypican-3 (GPC-3), heat shock protein 70 (HSP70) and cytokeratin 7 (CK7) in differentiating these lesions in FNA biopsies. Methods: Immunohistochemistry for these four markers was done in 45 HCCs, 22 metastatic carcinoma, 15 intrahepatic cholangiocarcinoma, 12 hepatocellular adenoma, 8 focal nodular hyperplasia, 12 large regenerative nodules arising in cirrhotic livers, and 10 specimens of normal liver tissues. Results: Arginase-1 reactivity was present in 43 of 45 HCCs (96.0%) and all of the benign liver lesions recruited in this study but not in any of MCs and ICCs (P < 0.001). GPC-3 stained 37 of 45 HCCs (82%) and only 1 of 22 MCs (5%), however, all cases of ICCs and the other benign lesions were negative for GPC-3 expression (P < 0.05). HSP70 showed markedly immunoreactivity in progressive tumors, 38 of 45 HCCs (84%), 18 of 22 MCs (82%), and 11 of 15 ICCs (73%), but was negative for all of the benign cases (P < 0.001). All ICCs showed diffused and strong immunostaining for CK7, but only 3 of 45 HCCs (7%) were detected to react with CK7. The combination of the four immunostaining markers for the diagnosis of HCC could raise the sensitivity and specificity to 98% and 100%, respectively. Conclusion: This study demonstrates that Arginase-1 is an extremely effective and specific immunohistochemistry marker for confirming hepatic origin, while GPC-3 and HSP70 is typically positive in malignant lesions. Our results indicated the accuracy of diagnosis can be enhanced by their combination of these four markers.

Keywords: Hepatocellular carcinoma, Arginase-1, Glypican-3, heat shock protein 70, cytokeratin 7, fine-needle aspiration

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant carcinoma, with a high mortality rate and increasing incidence worldwide [1]. HCC, which has a high prevalence in China, is the second leading cause of cancer-related deaths among males and is the third leading cause among females as a result of the high incidence of hepatitis B virus (HBV) infections [2]. Although various therapeutic options are available, the validity of these methods and the prognosis of patients with HCC remain extremely poor. However, the five-year survival rate can be increased to approximately > 70% when patients are diagnosed at an early stage [3]. Recently, much advancement has been achieved in radiological and imaging methods, such as ultrasound, computed tomography, and the magnetic resonance imaging detection of small lesions in the liver [4]. Nevertheless, the differentiation of nodular masses among HCCs and benign or metastatic lesions remains occasionally very difficult. Histopathological diagnosis remains as the golden standard for assessment [5]. For pathologists, distinguishing between low-grade HCC and benign nodular lesions can be very chal-
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Materials and methods

Clinical samples

A total of 124 paraffin-embedded FNAs of liver with their related clinical data were collected from the Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University from January 2012 to December 2013. The data consisted of 45 cases of HCC, 22 cases of MC, 15 cases of ICC, 12HCA, 8FNH, 12 large regenerative nodules arising in cirrhotic livers, and 10 specimens of normal liver tissues (NL). The diagnoses were reviewed by two gastrointestinal pathologists based on the histology, clinical data, and other assistant methods. The cases were staged and graded according to the Cancer Staging Manual, seventh edition, of the American Joint Committee on Cancer [9]. All the included cases were not treated with chemoembolization or systemic chemotherapy prior to needle biopsy. The primary HCC cases were classified into three groups according to the histological differentiation: 11 cases (24.4%) were well differentiated, 20 cases (44.4%) were moderately differentiated, and 14 cases (31.2%) were poorly differentiated. The MC cases consisted of 22 metastatic neoplasms, including 12 colon adenocarcinomas, 4 duodenum adenocarcinomas, 1 breast adenocarcinoma, 4 metastatic neuroendocrine carcinoma, and 1 pancreas ductal ade-
nocarcinoma. Informed consent was obtained from all the participants included in this work, and the use of the tissue specimens was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China).

Immunohistochemistry

The antibodies used in this study are listed in Table 1. Formalin-fixed and paraffin-embedded tissue sections were selected, and 4 μm sections were then dewaxed and rehydrated. The slides were treated with 3% H₂O₂ for 15 min to quench the endogenous peroxidase. Antigen retrieval was conducted by incubating the slides in 0.01 M citrate buffer (pH 6.0) at 100°C for 10 min. A standard immunohistochemical technique was then implemented using a Ventana Benchmark® XT autostainer (Ventana Medical SystemsInc., Tucson, AZ, USA).

The immunoreactivity of Arginase-1, GPC-3, HSP-70, and CK7 was scored by two pathologists. The percentage of reactive cells was quantified as 0 (no reactivity), 1 + (1%-10% staining), 2 + (10%-50% staining), or 3 + (> 50% staining). The intensity of immunostaining was scored as 0 (no staining), 1 + (weak staining), and 2 + (strong staining). The staining value (0-6+) was calculated as the intensity multiplied by the percentage of immunostained cells. A score of 0 indicated a negative value; a score of 1-2 + indicated low expression, and a score of 3-6 + indicated high expression. Only cytoplasmic reactivity or both cytoplasmic and nuclear reactivities were considered positive staining for Arginase-1 and HSP70. For GPC-3, positive staining was defined as coarsely granular cytoplasmic staining. Membrane stains were considered positive for CK7. Known positive and negative controls (without primary antibody) were used for each batch of slides.

Statistical analysis

Data were analyzed by SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). The χ² test was used to calculate the statistical significance of the variables. A P value of less than 0.05 was considered statistically significant.

Results

Summary of different antibody immunostaining patterns

The immunostaining results of the four antibodies in this work are summarized in Table 2. For Arginase-1, all benign liver lesions, including HCA, FNH, cirrhosis, and normal liver tissues, showed strong and diffuse immunostaining patterns. Moreover, the Arginase-1 antibody was positive in 43 of 45 (96%) HCCs, but none of the MC and ICC cases showed immunoreactivity with Arginase-1.

GPC-3 stained 37 of 45 (82%) HCCs and only 1 of 22 (5%) MCs (i.e., neuroendocrine carcinomas arising from the stomach). All cases of ICC and the other benign lesions were negative for GPC-3 expression.

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CK7 expression was observed in 3 of 45 (7%) HCCs and 3 of 22 (14%) MCs (i.e., 1 case of invasive breast carcinoma, 1 case of neuroendocrine carcinoma transferred from the pancreas, and 1 case of pancreas ductal carcinoma). No benign liver lesion was positive for CK7. However, CK7 showed strong expression in all 15 cases of ICC.

HSP70, which was absolutely negative in all cases of benign liver masses and tissues,
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Figure 1. HCC. A. Well differentiated HCC with only focally thickened cell plates and mild cytologic atypia. This morphology could overlap with many benign liver mass lesions (hematoxylin and eosin, × 100), B. Arginase-1 shows strong, diffuse positivity in cell nuclear and cytoplasm (immunohistochemical stain, × 100), C. GPC-3 also shows moderate-strong, diffused positivity in cell cytoplasm (immunohistochemical stain, × 200), D. The same case with A with magnification × 200 (hematoxylin and eosin), E. HSP70 also shows strong, diffused positivity in cell nuclear and cytoplasm (immunohistochemical stain, × 100), F. CK7 showed no immunoreactivity in HCC (immunohistochemical stain, × 100).

Figure 2. MC. A. Fine-needle biopsy of hepatic metastasis of breast carcinoma made by H&E sections (× 100), B. Arginase-1 showed no immunoreactivity in MC (× 400), C. GPC-3 showed no immunoreactivity in MC (× 400), D. Fine-needle biopsies of breast carcinoma made by H&E sections (× 400), E. Diffused and strong nuclear and cytoplasmic immunostaining for HSP70 (× 100), F. CK7 shows strong, diffuse positivity in membrane (immunohistochemical stain, × 400).

showed apparent immunoreactivity in 38 of 45 (84%) HCCs, 18 of 22 (82%) MCs, and 11 of 15 (73%) ICCs. Among the 18 MC cases, 10 originated from colon carcinoma, 3 from duodenal
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carcinoma, 1 from invasive breast carcinoma, 3 from neuroendocrine carcinoma, and 1 from pancreas ductal carcinoma.

Comparison of the immunoreactivity of different antibodies in malignant and benign liver nodular lesions

To further investigate the immunoreactivities of the four markers in differentiating HCCs from other malignant or benign liver lesions in the FNA samples, we analyzed the sensitivity, specificity, and predictive value between HCCs and other cases.

In Table 3, Arginase-1 and GPC-3 immunoreactivity were diffused and strong in the majority of the HCCs (Figure 1) and showed high specificity and positive predictive value for distinguishing HCCs from MCs ($P < 0.001$). None of the MC cases showed immunoreactivity for Arginase-1; only 1 of 22 in the MC cases was positive for GPC-3. The combination of GPC-3 and HSP70 was also useful because the specificity and positive predictive value reached 100% when both GPC-3 and HSP70 were positive ($P < 0.001$).

Arginase-1 stained most of HCCs (43 of 45) and all 12 cases of HCA. By contrast, CK7 had no immunoreactivity in HCCs (Figure 3) and stained only a minority of HCCs (3 of 45). Compared with HCCs, 37 of 45 HCCs and 38 of 45 HCCs showed an extreme increase in GPC-3 and HSP70 expression, respectively ($P <
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Table 5. Comparison of the immunoreactivity of different antibodies between HCC and ICC

<table>
<thead>
<tr>
<th></th>
<th>Arginase-1</th>
<th>GPC-3</th>
<th>CK7</th>
<th>HSP70</th>
<th>GPC-3 or HSP70</th>
<th>GPC-3 and HSP70</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC (positive/cases)</td>
<td>43/45</td>
<td>37/45</td>
<td>3/45</td>
<td>38/45</td>
<td>44/45</td>
<td>31/45</td>
</tr>
<tr>
<td>ICC (positive/cases)</td>
<td>0/15</td>
<td>0/15</td>
<td>15/15</td>
<td>11/15</td>
<td>11/15</td>
<td>0/15</td>
</tr>
<tr>
<td>(P) (HCC vs. ICC)</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001*</td>
<td>0.335</td>
<td>0.003*</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Sensitivity for HCC (%)</td>
<td>96</td>
<td>82</td>
<td>7</td>
<td>84</td>
<td>98</td>
<td>69</td>
</tr>
<tr>
<td>Specificity for HCC (%)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>27</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>100</td>
<td>100</td>
<td>17</td>
<td>78</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>88</td>
<td>65</td>
<td>0</td>
<td>36</td>
<td>80</td>
<td>52</td>
</tr>
</tbody>
</table>

*\(P < 0.05\).

As described above, Arginase-1 showed diffused and strong immunoreactivity in 43 of 45 HCCs and all combination cases (Figures 5, 6), including benign liver lesion masses and normal liver tissues (\(P > 0.05\)). However, in contrast to the high expression rate in HCC, no cases were positive for GPC-3 and HSP70 immunostaining in the combination groups (\(P < 0.001\)). GPC-3 and/or HSP70 showed 100% specificity and positive predictive value in differentiating HCCs from benign and normal liver tissues (Table 6).

In summary, Arginase-1 is a sensitive marker for tissues originating from the liver, including HCC, HCA, FNH, cirrhosis, and NL tissues, whereas none of the MC or ICC cases were pos-

0.001). Either GPC-3 and HSP70 and their combination showed 100% specificity and positive predictive values. The sensitivity was 98% when only one marker was positive (Table 4).

As shown in Table 5, CK7 demonstrated positive immunoreactivity in all 15 (100%) cases of ICC (Figure 4), and only 3 of 45 cases of HCC showed positive immunoreactivity for CK7, with focal and weak staining. By contrast, Arginase-1 and GPC-3 showed significantly increased sensitivity and specificity for the diagnosis of HCCs (96%, 100% vs. 82%, 100%). Given that the staining rates for HCC and ICC were high, HSP70 can not differentiate HCCs from ICCs (\(P > 0.05\)).

Figure 4. ICC. A. A cell block section of moderate to low differentiated ICC (hematoxylin and eosin, \(\times\) 100), B. Negative immunostaining for Arginase-1 (\(\times\) 100), C. Negative immunostaining for GPC-3 (\(\times\) 100), D. The same cell block section of ICC (hematoxylin and eosin, \(\times\) 400), E. Diffused and strong nuclear and cytoplasmic immunostaining for HSP70 (\(\times\) 100), F. Moderate to strong, diffuse positivity for CK7 (immunohistochemical stain, \(\times\) 400).
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Moreover, 37 among the 45 HCC patients presented an elevated level of immunostaining for GPC-3, and this finding indicated that the expression of GPC-3 may be useful for the diagnosis of HCC. HSP70 showed a high expression rate (38 of 45) in HCCs and was upregulated in MCs (18 of 22) and ICCs (11 of 15). However, all cases of HCA and combination groups were negative for HSP70. Thus, the positive staining of HSP70 was valuable to dif-

Figure 5. FNH. A. Typical hematoxylin and eosin pattern of biopsies of FNH subtypes (× 100), B. Diffused nuclear and cytoplasmic immunostaining for Arginase-1 (× 200), C. Negative immunostaining for GPC-3 (× 400), D. The same cell block section of FNH (hematoxylin and eosin, × 200), E. Negative immunostaining for HSP70 (× 200), F. Negative immunostaining for CK7 (× 400).

Figure 6. Regenerative Nodules in Liver Cirrhosis. A. Fine-needle biopsy of a case of regenerative nodules in liver cirrhosis made by H&E sections (× 100), B. Diffused nuclear and cytoplasmic immunostaining for Arginase-1 in regenerative liver cells, C. Negative immunostaining for GPC-3 (× 400), D. The same cell block section of liver cirrhosis (hematoxylin and eosin, × 400), E. Negative immunostaining for HSP70 (× 200), F. Negative immunostaining for CK7 in liver cells, but positive in small bile duct (× 400).
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Table 6. Comparison of the immunoreactivity of different antibodies between HCC and combination

<table>
<thead>
<tr>
<th></th>
<th>Arginase-1</th>
<th>GPC-3</th>
<th>CK7</th>
<th>HSP70</th>
<th>GPC-3 or HSP70</th>
<th>GPC-3 and HSP70</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCC (positive/cases)</strong></td>
<td>43/45</td>
<td>37/45</td>
<td>3/45</td>
<td>38/45</td>
<td>44/45</td>
<td>31/45</td>
</tr>
<tr>
<td><strong>Combination (positive/cases)</strong></td>
<td>30/30</td>
<td>0/30</td>
<td>0/30</td>
<td>0/30</td>
<td>0/30</td>
<td>0/30</td>
</tr>
<tr>
<td><strong>P (HCC vs. Combination)</strong></td>
<td>0.242</td>
<td>&lt; 0.001*</td>
<td>0.149</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td><strong>Sensitivity for HCC (%)</strong></td>
<td>96</td>
<td>82</td>
<td>7</td>
<td>84</td>
<td>98</td>
<td>69</td>
</tr>
<tr>
<td><strong>Specificity for HCC (%)</strong></td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Positive predictive value (%)</strong></td>
<td>59</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Negative predictive value (%)</strong></td>
<td>0</td>
<td>79</td>
<td>42</td>
<td>81</td>
<td>97</td>
<td>68</td>
</tr>
</tbody>
</table>

*P < 0.05.

Discussion

HCC is the fifth most common cancer and the second leading cause of cancer-related deaths worldwide [1]. Most patients with HCC are diagnosed at an advanced stage in their illness, and the prognosis is generally poor [10]. Currently, AFP measurement and ultrasound detection are widely and routinely used to screen HCC in China. Nevertheless, the level of serum AFP is not always specific for HCC because upregulated AFP is also detected in patients with viral hepatitis [11, 12]. Under these circumstances, FNA biopsy is necessary for HCC identification, especially when the imaging findings are atypical.

Notably, the clinical application of percutaneous FNA biopsy has been proposed as a safe, effective, and minimally invasive procedure for the diagnosis of liver masses [13]. The sensitivity and specificity of FNA for detecting liver malignancy are around 90% and 100%, respectively [14]. Although morphological observation is often available for pathologists, arrive at a precise final diagnosis remains challenging because of the limited size of samples with core biopsies and the mimicking of histopathological features between liver lesions. Thus, immunohistochemical markers may play a very important role in clinically atypical and indeterminate cases. Recently, a series of diagnostically available immunohistochemical markers for the identification of HCC was applied in routine surgical pathology practice, however, the value of each marker is attributed to tumor heterogeneity and the insufficient sensitivity of each marker. In this work, we proposed Arginase-1, GPC-3, CK7, and HSP70 as an immunohistochemistry biomarker panel for routine diagnostic FNA biopsy and evaluated the specificity and positive predictive value of these markers.

Arginase-1 is a key enzyme of the urea cycle and is found in the liver, this enzyme catalyzes the conversion of L-arginine into L-ornithine and urea. In the past few years, Arginase-1 has been described as a potential and valuable immunohistochemical marker for differentiating HCCs from other tumors [15, 16]. In our study, Arginase-1 demonstrated diffused and strong reactivity in most HCC cases and all benign liver masses, with no immunoreactivity against MC and ICC. However, several studies demonstrated Arginase-1 immunoreactivity in a small portion of pancreatic adenocarcinomas, ICCs, and prostatic adenocarcinomas. Fortunately, all the cases were negative for GPC-3 [6, 16]. The sensitivity for Arginase-1 in HCCs was 96%, and the positive predictive value between HCCs from MCs and ICCs was 100%.

GPC-3 is an oncofetal antigen and a member of the glypcan family of glycosyl phosphatidylinositol-anchored cell-surface heparin sulfate proteoglycans. Wang summarized the studies on GPC-3 from 2001 to 2014 and concluded that GPC-3 plays a crucial role in HCC cell proliferation and metastasis. Moreover, GPC-3 is also involved in signaling pathways during hepatocyte malignant transformation [17].
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A flow chart for the clinical practical procedure in liver biopsy diagnosis.

Figure 7. A flow chart for the clinical practical procedure in liver biopsy diagnosis.

Percentage of GPC-3 expression reported in HCCs ranges from 49% to 97.7%. In our work, GPC-3 was expressed in 37 (82%) HCCs, which is consistent with the results of previous studies [18]. Although GPC-3 positivity was observed in a subset of yolk sac tumor, choriocarcinoma, and melanoma, only one case of neuroendocrine carcinoma arising from the stomach showed immunoreactivity against GPC-3 in our MC group [18, 19]. Moreover, the sensitivity of GPC-3 for identifying HCCs was 82%, whereas the positive predictive value between HCCs and the other groups was 100%. Therefore, the immunoreactivity of GPC-3 can significantly distinguish HCCs from other liver masses in FNA biopsy detection.

Over the past few years, the elevation of HSP70 has been assessed in various solid carcinomas [20]. HSP70 may play an important role in protecting cells from DNA damage and perform anti-apoptotic functions. In our series of experiments [21, 22], HSP70 stained 84% of HCC, 82% of MC, and 73% of ICC, without immunoreactivity to benign lesions. Thus, HSP70 can serve as a potential marker of malignancy, although it cannot differentiate HCCs from MCs or ICCs.

CK7 is an effective immunohistochemical diagnostic tool for the study of the origins of tumors from normal epithelial tissues and cancers, especially in adenocarcinoma from the ovary, breast, lung, and bile duct [23, 24]. The utility of CK7 in this work is as a supplementary tool for differentiating HCCs from other liver masses. Thus, we propose Arginase-1, GPC-3, HSP70, and CK7 to comprise a marker panel for routine diagnostic work. (1) Arginase-1 is a reliable marker for confirming the histogenesis of liver cells because of its high specificity and sensitivity. (2) The positive staining of GPC-3 and HSP70 can distinguish malignant components from benign lesions, whereas the negative results do not always indicate benign outcomes. The combination of GPC-3 and HSP70 was also evaluated in our work, and the interpretation of the immunostaining results is an integrated procedure under actual conditions. (3) We considered CK7 to be a vital complementary marker in diagnosis because of the high specificity and sensitivity in ICC differentiation. The positive staining results also prompt the presumption for MC.

The diagnostic differentiation of liver masses is occasionally challenging, especially in FNA samples. On the basis of our results, a flow chart (Figure 7) is presented for the clinical practical procedure in liver biopsy diagnosis, which also shows the possible outcomes of potential staining. Further analysis is needed to search for novel biomarkers for HCC and more effective treatment strategies.

Acknowledgements

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References


in C3H 10T1/2 cells and protects from DNA damage. Toxicol In Vitro 2003; 17: 561-566.


