Original Article
HCRP1, ID4 and Glypican-3: an optimal panel of biomarkers for diagnosis of hepatocellular carcinoma

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Abstract: Background: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with high morbidity and mortality. The aim of this study was to assess the diagnostic role of HCC related protein 1 (HCRP1) and inhibitor of DNA Binding 4 (ID4) as novel reliable markers for HCC diagnosis. Methods: Immunohistochemistry for HCRP1, ID4 and Glypican-3 (GPC-3) was performed in 98 cases of HCCs, 15 large regenerative nodules arising in cirrhotic livers, 12 hepatocellular adenomas (HCA), 10 focal nodular hyperplasias (FNH), and 20 specimens of normal liver tissues (NL). Results: HCRP1 immunoactivity was decreased in 64 of 98 (65.3%) HCC cases but present in almost all of the benign liver nodules (56/57, 98.2%, $P < 0.001$). 68 of 98 (69.4%) and 70 of 98 (71.4%) HCC cases were positive for ID4 and GPC-3, respectively, which were much higher than in benign lesions. Even though HCRP1 is highly specific (98.25%) in differentiating well differentiated HCC (WDHCC) from benign liver nodules, it has only a limited value because of its low sensitivity (37.5%), neither for the ID4, GPC-3 alone or combination ($P > 0.05$). The expression of HCRP1 alone could efficiently distinguish WDHCC from moderate-poorly differentiated HCC (M-PHCC), and the combination of using either two or three markers could notably increase the diagnosis accuracy ($P < 0.05$). Conclusion: HCRP1 and ID4 represent potentially novel valuable biomarkers for distinguishing HCC from benign liver nodules, and it is recommended to use the combination of HCRP1, ID4 and GPC-3 as a panel in HCC differentiation estimation.

Keywords: HCRP1, ID4, Glypican-3, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent human cancers worldwide, especially in China. This can be attributed to the high incidence of hepatitis B viral infections [1]. The mortality rate of HCC patients remains extremely high, but we believe early detection and diagnosis could increase the five-year survival rate approximately > 70% if the lesion could be detected and diagnosed at an earlier stage [2]. Despite the great progress that has been made in the renovation of radiological and imaging methods such as ultrasound, computed tomography, and magnetic resonance imaging detection, the differentiation of nodular masses among HCC and benign tumors remains occasionally very difficult. It is especially difficult to discern between HCC from dysplastic nodules, even for pathologists, who are the final diagnostic reporter representing the gold standard [3].

Hitherto, a range of various biomarkers have been used to distinguish HCCs from other mimick liver nodule lesions, including α-fetoprotein (AFP), Arginase-1, hepatocyte paraffin antigen-1 (HepPar-1), glypican-3 (GPC-3), glutamine synthetase (GS), heat shock protein 70 (HSP 70), and the enhancer of zeste homologue 2 [4-6]. However, the sensitivity and specificity of the individual markers for proper diagnosing is barely satisfactory and may influence the accuracy of the diagnosis and subsequent therapy [7]. Therefore, there is an urgent clinical need to develop novel reliable biomarkers to assist the differential diagnosis between HCCs and other mimic lesions.
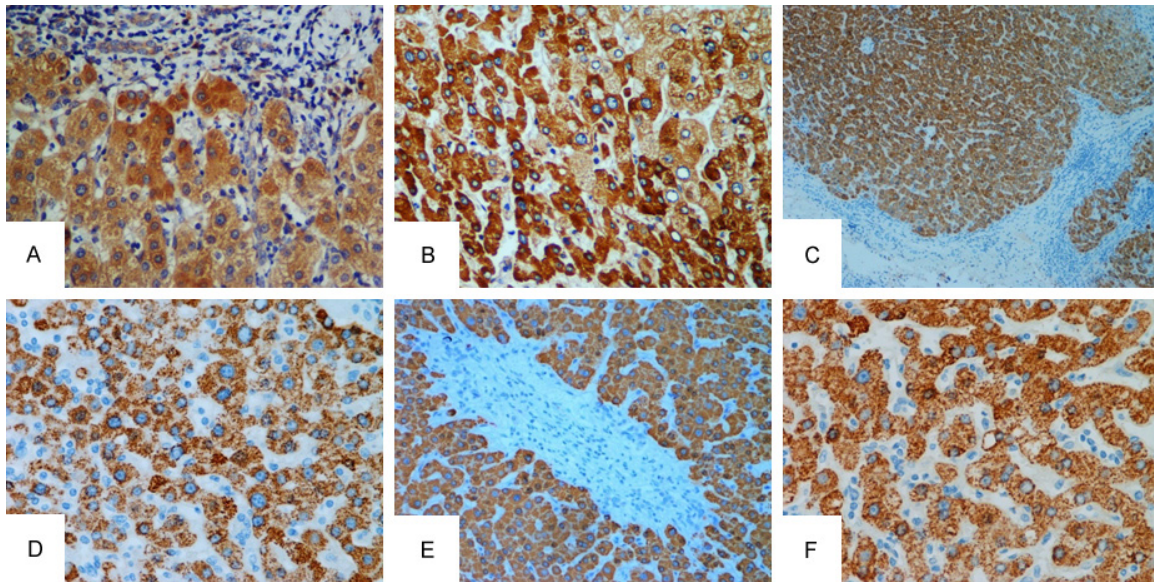
Figure 1. Cytoplasmic HCRP1 staining in samples of WDHCC (A, ×400), M-PDHCC (B, ×200), LC (C, ×100), HCA (D, ×400), FNH (E, ×200), NL (F, ×400).

Table 1. Expression of HCRP1 in different lesions

Disease Group	Patients, n	HCRP1 expression, n (%)		χ^2	P-value
		Positive	Negative		
HCC	98	34 (34.7%)	64 (65.3%)	59.978	< 0.001*
LC	15	14 (93.3%)	1 (6.7%)		
HCA	12	12 (100.0%)	0 (0%)		
FNH	10	10 (100.0%)	0 (0%)		
NL	20	20 (100.0%)	0 (0%)		

*$P < 0.05$.

HCC related protein 1 (HCRP1) and inhibitor of DNA Binding 4 (ID4) are newly discovered genes that are involved in the HCC malignant process, particularly in the regulation of proliferation and migration [8]. Our previous study also predicted HCRP1 to be a valuable prognostic factor involved in acquisition of the mesenchymal phenotype of HCC cells and significantly promote the metastasis of HCC [9]. Zhang proved ID4 could promote cell proliferation and colony formation capability in HCC cells [10]. The utility of the two novel proteins in clinical diagnosis differentiation was never mentioned.

In the present study, we therefore analyzed the expression patterns of HCRP1, ID4 and acknowledged HCC marker Glypican-3 among several differentiated HCCs and benign module lesions. We determined the accuracies of different panels of these markers in differential diagnosis between well differentiated HCC (WDHCC) and moderate-poorly differentiated HCC (M-PHCC) and benign lesions, respectively.

Materials and methods

Clinical samples

A total of 135 formalin-fixed paraffin-embedded tissues of liver nodules were randomly selected retrospectively from patients who underwent curative resection from the Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University between January 2011 and December 2015. There were 98 cases of HCC, 15 large regenerative nodules arising in cirrhotic livers, 12 hepatocellular adenomas (HCA), 10 focal nodular hyperplasias (FNH), and 20 specimens of normal liver tissues (NL). The pathological diagnoses were reviewed by two experienced pathologists (XU JW and Xu YL). Tumor stage was defined according to the Cancer Staging Manual, seventh edition, of the American Joint Committee on Cancer [11]. The primary HCC cases were classified into three groups according to the histological differentiation: 16 cases (16.3%) were well differentiated, 45 cases (45.9%) were moderately differentiated, and 37 cases (37.8%) were poorly differentiated. This study was approved by the Research Ethics
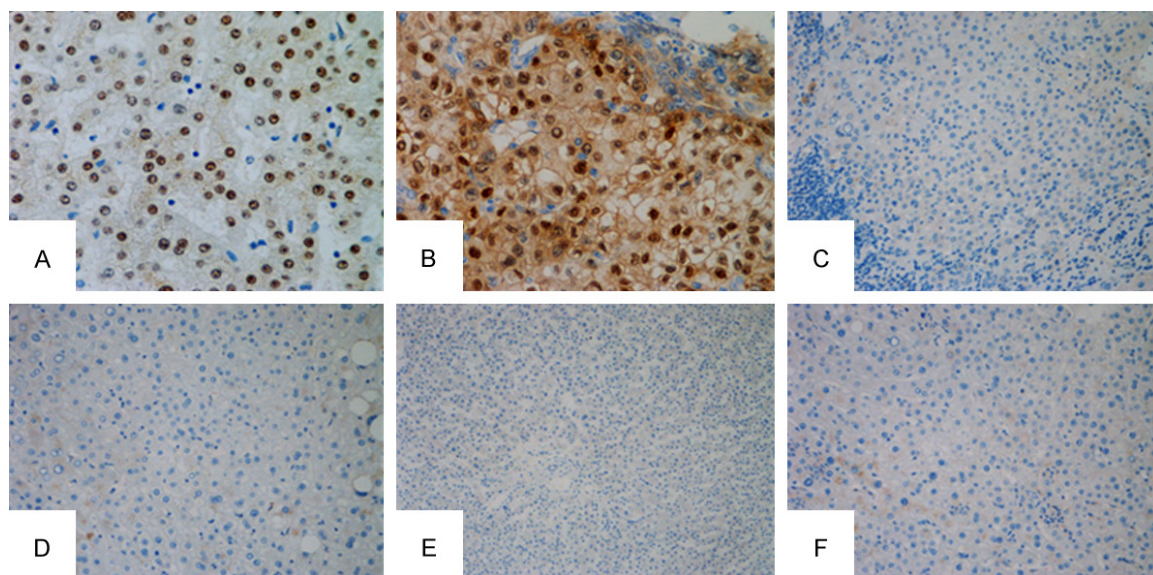
Figure 2. Nuclear ID4 staining in samples of WDHCC (A, ×400), M-PDHCC (B, ×400), LC (C, ×200), HCA (D, ×200), FNH (E, ×100), NL (F, ×200).

Table 2. Expression of ID4 in different lesions

Disease Group	Patients, n	ID4 expression, n (%)		χ^2	P-value
		Positive	negative		
HCC	98	68 (69.4%)	30 (30.6%)	53.127	< 0.001*
LC	15	4 (26.7%)	11 (73.3%)		
HCA	12	2 (16.7%)	10 (83.3%)		
FNH	10	0 (0%)	10 (100.0%)		
NL	20	0 (0%)	100.0%		

*P < 0.05.

Committee of Shandong Provincial Hospital Affiliated to Shandong University.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections were selected, and 4 μm sections were then deparaffinized in xylene and rehydrated using a series of graded washes with ethanol. The slides were treated with 3% H_2O_2 for 15 min to quench the endogenous peroxidase. Antigen retrieval was performed 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 Systems Inc., Tucson, AZ, USA). Negative control slides omitting the primary antibodies were conducted for all assays.

Evaluation and scoring

A semi-quantitative scoring system considering both intensity of staining and percentage of tumor cells was applied, the staining value (0-9) was calculated as the intensity (negative = 0; weak = 1; moderate = 2; strong = 3) multiplied by the percentage (< 10% = 1; 11%-50% = 2; > 51% = 3). The intensity of the staining was assessed in 100 cells. Positive cell staining was defined by cases with staining scores ≥ 2 for the purposes of this study.

Statistical analysis

SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The χ^2 test was used to calculate the statistical significance of the variables. A P value of less than 0.05 was considered significant.

Results

HCRP1 expression was decreased in HCC

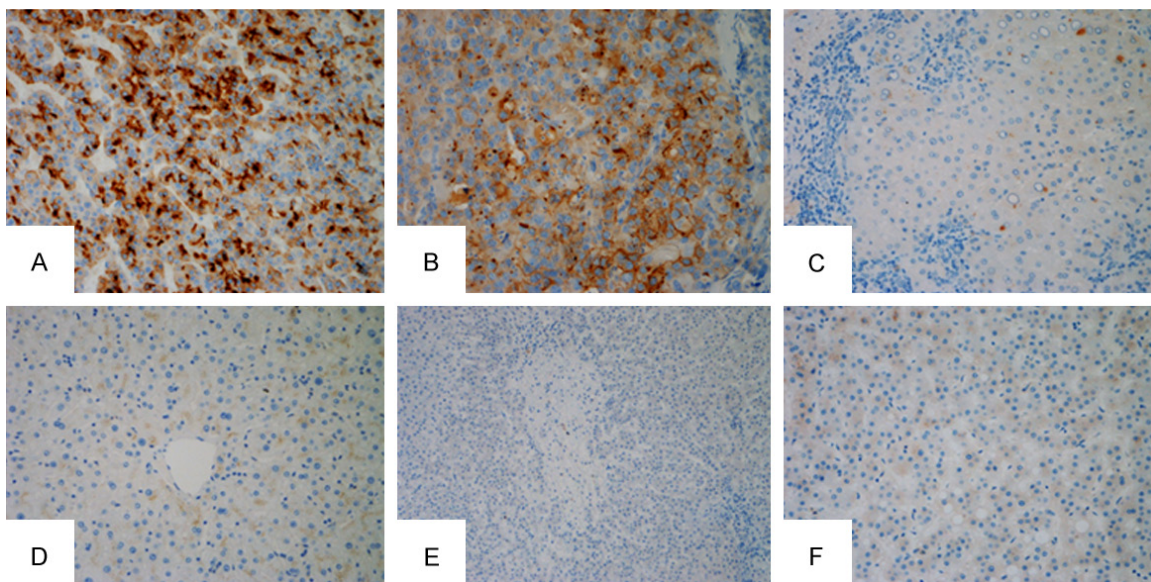34 (34.7%) of 98 HCC cases were positive for HCRP1, while 14 of 15 (93.3%) LC and all cases

Figure 3. Cytoplasmic GPC-3 staining in samples of WDHCC (A, ×400), M-PDHCC (B, ×400), LC (C, ×200), HCA (D, ×200), FNH (E, ×100), NL (F, ×200).

Table 3. Expression of GPC-3 in different lesions

Disease Group	Patients, n	GPC-3 expression, n (%)		x^2	P-value
		Positive	negative		
HCC	98	70 (71.4%)	28 (28.6%)	64.160	< 0.001*
LC	15	2 (13.3%)	13 (86.7%)		
HCA	12	0 (0%)	12 (100.0%)		
FNH	10	1 (10.0%)	9 (90.0%)		
NL	20	0 (0%)	20 (100.0%)		

*P < 0.05.

of HCA (12 of 12), FNH (10 of 10) and NL (20 of 20) showed diffuse immunostaining for HCRP1. The immunostaining in these cases was granular and cytoplasmic (**Figure 1**). HCRP1 is a useful marker in differentiating HCC from benign liver nodule and normal liver tissues (**Table 1**, P < 0.001).

ID4 expression is extremely upregulated in HCC

Of 98 HCCs, 68 cases (69.4%) were diffuse and strongly demonstrated ID4 nuclear positivity, whereas only 4 of 15 and 2 of 12 were positive in LC and HCA (**Figure 2**), respectively. No cases of FNH and NL showed immunoreactivity for ID4 (**Table 2**, P < 0.001).

Glypican-3 expression is extremely upregulated in HCC

The cytoplasmic immunoreaction of Glypican-3 was seen in most HCC cases (70/98, 71.4%).

Only 2 of 15 and 1 of 10 showed immunostaining in LC and FNH (**Figure 3**), respectively. Furthermore, there was no expression of GPC-3 in HCA and NL (**Table 3**, P < 0.001).

The expression of different markers between WDHCC and benign lesions

In 16 WDHCC cases, 6 showed significantly decreased HCRP1 expression, while only 1 of 57 was negative for HCRP1 in WDHCC. 6 of 16 ID4 and 9 of 16 GPC-3 showed positive immunostaining in WDHCC and 6 of 57 and 3 of 57 in benign lesions, respectively. The combination of ID4+GPC-3 or HCRP1+ID4+GPC-3 stained 10 of 16 WDHCC, but the PPV was only 52.63% and 50.00% (**Table 4**). Statistically, although HCRP1 showed the highest specificity (98.25%) in all of the three markers alone or in combination, the expression of HCRP1 (AUC = 0.562, 95 CI: 0.468-0.655, P = 0.199) showed no significantly different value of discrimination between WDHCC and benign lesions, neither for ID4 (AUC = 0.527, 95 CI: 0.4336-0.6212, P = 0.568) and GPC-3 (AUC = 0.527, 95 CI: 0.4336-0.6212, P = 0.568) alone, two markers (HCRP1+ID4, AUC = 0.5, 95 CI, 0.406-0.594, P = 1.000); (HCRP1+GPC-3, AUC = 0.521, 95 CI: 0.4267-0.6144, P = 0.668); (ID4+GPC-3, AUC = 0.521, 95 CI: 0.4267-0.6144, P = 0.668); or

Table 4. Diagnostic effects of different markers on highly differentiated HCC and benign lesions

	WDHCC (*n* = 16)	Benign lesions (*n* = 57)	Sen	Spe	PPV	NPV
HCRP1 (-)	6	1	37.50%	98.25%	85.71%	84.85%
ID4 (+)	6	6	37.50%	89.47%	50.00%	83.61%
GPC-3 (+)	9	3	56.25%	94.74%	75.00%	88.52%
HCRP1+ID4	9	7	56.25%	87.72%	56.25%	87.72%
HCRP1+GPC-3	9	4	56.25%	92.98%	69.23%	88.33%
ID4+GPC-3	10	9	62.50%	84.21%	52.63%	88.89%
HCRP1+ID4+GPC-3	10	10	62.50%	82.46%	50.00%	88.68%
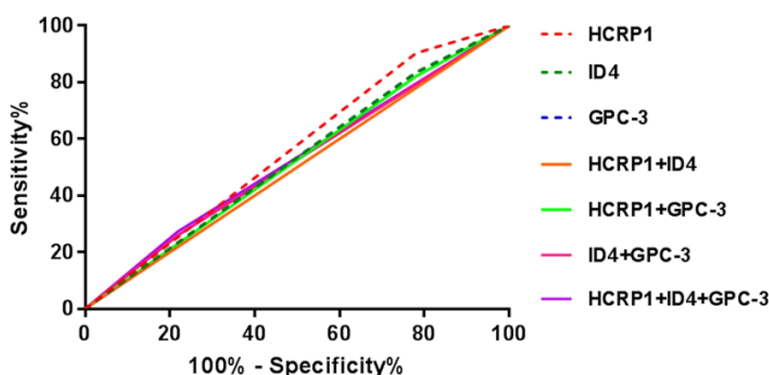
Figure 4. ROC curve analysis of individual markers and combinations of HCRP, ID4, and GPC-3 for discriminating between HCC and benign lesions.

three markers combination (HCRP1+ID4+GPC-3, AUC = 0.527, 95 CI: 0.4336-0.6212, P = 0.568, **Table 4**; **Figure 4**).

The expression of different markers between WDHCC and M-PDHCC

As the degree of differentiation of HCC is significantly associated with staging, therapeutic regimen tailing and prognosis, it is of great importance to seek out a useful marker in clinical practice to cover the shortage of morphological determination. 24 of 82, 62 of 82 and 61 of 82 M-PDHCC showed strong to moderate positivity for HCRP1, ID4 and GPC-3, whereas 10 of 16, 6 of 16 and 9 of 16 showed immunostaining for HCRP1, ID4 and GPC-3 in WDHCC, respectively. Only HCRP1 expression revealed distinct significance in differentiation between WDHCC and M-PDHCC (AUC = 0.592, 95 CI: 0.512-0.671, P = 0.026), and ID4 (AUC = 0.571, 95 CI: 0.4912-0.6516, P = 0.084) and GPC-3(AUC = 0.561, 95 CI: 0.4808-0.6416, P = 0.139) alone did not differ between the groups. However, the combination of using either two or three markers could notably increase the diag-

nostic accuracy. (HCRP1+ID4, AUC = 0.679, 95 CI: 0.6029-0.7543, P < 0.001; HCRP1+GPC-3, AUC = 0.658, 95 CI: 0.5813-0.7350, P < 0.001; ID4+GPC-3, AUC = 0.648, 95 CI: 0.5706-0.7254, P < 0.001; HCRP1+ID4+GPC-3, AUC = 0.725, 95% CI: 0.6521-0.7969, P < 0.001, **Table 5**; **Figure 5**). In addition, HCRP1 and ID4 combination showed the highest sensitivity (81.25%) and negative predictive values (93.62%), which is better than the acknowledged marker GPC-3, indicating the potential application value in routine clinical work.

Discussion

HCRP1, which is also known as the homologue of vacuolar protein sorting 37A (hVps37A), is located in a high-frequency LOH region of chromosome 8p22-23 [12]. Recently, downregulation of HCRP1 in several tumors as oral and oropharyngeal cancer, non-small cell lung cancer, gastric cancer, prostate cancer, and glioma were reported and associated with worse outcomes [13-17], indicating that HCRP1 is capable of being a prognostic indicator in tumors. We have reported that HCRP1 expression is significantly decreased in breast cancer and is correlated with shorter survival due to enhancement of EGFR phosphorylation [18, 19]. In HCC, downregulation of HCRP1 was reported in our previous study to be involved in promoting HCC cell proliferation, migration, and invasion via EMT and activating EGFR pathways [9, 20]. Similarly, lower HCRP1 expression was also correlated with shorter relapse-free *survival* and overall *survival* and decreased HCRP1 level is

Table 5. Diagnostic effects of different markers between WDHCC and M-PDHCC

	WDHCC (n = 16)	M-PDHCC (n = 82)	Sen	Spe	PPV	NPV
HCRP1 (+)	10	24	62.50%	70.73%	29.41%	90.63%
ID4 (-)	10	20	62.50%	75.61%	33.33%	91.18%
GPC-3 (-)	7	21	43.75%	74.39%	25.00%	87.14%
HCRP1+ID4	13	38	81.25%	53.66%	25.49%	93.62%
HCRP1+GPC-3	10	37	62.50%	54.88%	21.28%	88.24%
ID4+GPC-3	11	34	68.75%	58.54%	24.44%	90.57%
HCRP1+ID4+GPC-3	13	47	81.25%	42.68%	21.67%	92.11%
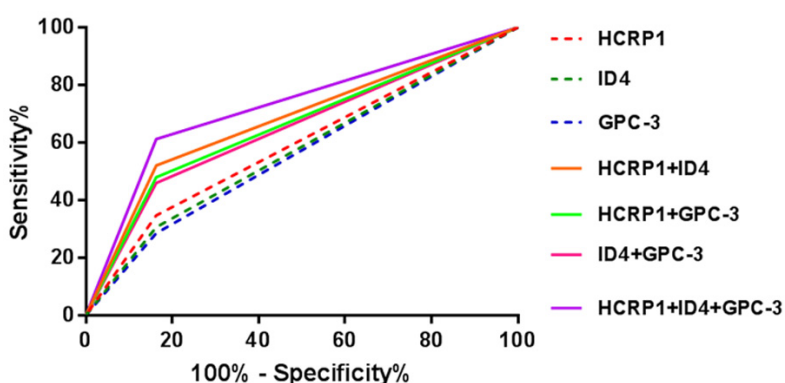
Figure 5. ROC curve analysis of individual markers and combinations of HCRP, ID4, and GPC-3 for discriminating between WDHCC and M-PDHCC.

an independent prognostic marker in HCC patients [21]. In this work, 34 of 98 HCC cases showed moderate to strong positivity for HCRP1, while HCRP1 showed diffuse and strong immunostaining in 14 of 15 (93.3%) LC and all of the other benign cases including HCA (12 of 12), FNH (10 of 10) and 20 cases of NL. Therefore, HCRP1 can be used as a valuable marker to distinguish HCC from benign liver nodules and normal liver tissues.

Inhibitor of DNA binding factors (ID1-ID4) contain a highly conserved helix-loop-helix dimerization domain through which they form heterodimers with basic helix-loop-helix transcription factors [22]. It is reported that IDs are involved in numerous cell processes, including cell proliferation, differentiation, and tumorigenesis [23]. In cancer, the role of ID4 was not certain on the basis of different tumor entities, even in the same organs. It appears to act as a tumor suppressor in most cancers such as colorectal cancer [24], gastric cancer [25], prostate cancer [26], lymphoma [27] and lung cancer due to epigenetic silencing. However,

ID4 was also reported to behave as a tumor promoter in several cancers such as triple negative breast cancer [28], bladder cancer [29] and glioma [30]. In HCC, Zhang reported ID4 expression was overexpressed in most HCC patients and ID4 could promote HCC cell proliferation, clonogenicity and tumorigenicity, but overexpression of CCAAT/enhancer-binding protein β could efficiently inhibit Id4 expression in vitro [10]. In our study, we detected 68 of 98 cases of positive immunostaining for ID4, while only 4 of 15 and 2 of 12 were positive in LC and HCA, respectively. None of the cases of FNH and NL showed immunoreactivity for ID4, indicating ID4 immunoactivity could be developed as a potent biomarker in liver nodular disease differentiation.

GPC3, a member of the glypican family of glycosylphos-phatidylinositol-anchored cell surface heparan sulfate proteoglycans, is known to show overexpression in HCC than in normal liver tissues [31]. The upregulation of GPC-3 is already used in routine clinical diagnostic procedures in differentiation of HCC from other liver nodular masses [32]. In recent years, treatment of HCC with anti-GPC3 immunotoxins represents a new therapeutic option [33, 34]. In our study, Glypican-3 immunostaining was seen in 70 of 98 HCC cases, but only 2 of 15 and 1 of 10 were positive for Glypican-3 in LC and FNH, respectively. However, there is no case expressing GPC-3 in HCA and NL.

Discriminating HCC from benign liver masses is of great importance in clinical work. We were satisfied that all three markers were effective. Although numerous strategies have been improved to detect WDHCC at an early stage, the distinction between WDHCC and benign lesions remains challenging in both clinical and pathological diagnosis, even to experienced hepatic clinicians and hepatopathologists due to the vitality of choosing optimal treatment methods. To our disappointment, although the specificity of HCRP1(-) and GPC-3(+) was 98.25% and 94.74%, respectively, neither one marker alone nor the combination of any two or three markers showed no significance.

The treatment of HCC is based on the histologic grade and pathologic stage, which are majorly defined by the degree of differentiation. HCC patients at early-stage HCC with well differentiated histological features can undergo surgical resection or radiofrequency ablation with favorable prognosis [35, 36]. However, patients at intermediate-late stage with moderate to poor differentiation are only eligible for radiotherapy or chemotherapy, and poor outcomes are likely. Therefore, to accurately identify the differentiation is greatly demanded in clinical practice, especially in biopsy specimens. In our study, the expression of HCRP1 was significantly decreased in M-PHCC compared to WDHCC. Although ID4 and GPC-3 expression did not effectively differentiate WDHCC from M-PHCC independently, the combination of two or three markers could distinguish WDHCC from M-PHCC effectively.

In conclusion, we suggest a new immunohistochemical panel for the discrimination of HCC from benign liver masses containing HCRP1, ID4 and GPC-3. The combination of these three markers was useful in distinguishing between WDHCC and M-PHCC, and this may be of great importance in directing clinical treatment.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Jiawen Xu, Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University, No. 324 Weiqi Avenue, Jingwu Road, Jinan 250021, Shandong, PR China. Tel: +86-531-68776430; Fax: +86-531-87037504; E-mail: 4366203@qq.com

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