Oncology and Translational Medicine
DOI 10.1007/s10330-019-0351-1

August 2019, Vol. 5, No. 4, P153–P161

ORIGINAL ARTICLE

Immunohistochemical panel of glypican-3, hepatocyte paraffin antigen-1, arginase-1, cytokeratin-19, and human epithelial membrane antigen for the differential diagnosis of liver tumors*

Dongling Zhu[1], Dong Kuang[2], Sijuan Zou[1], Lixing Chen[1], Yuanli Zhu[2], Xiaohua Zhu[1] (✉)

[1] Department of Nuclear Medicine and PET, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[2] Department of Pathology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Abstract

Objective Clinical immunohistochemistry plays an increasingly important role in pathologic diagnosis. We investigated the usefulness of an immunohistochemical panel of glypican-3 (GPC3), hepatocyte paraffin antigen-1 (HepPar-1), arginase-1 (Arg-1), cytokeratin-19 (CK19), and human epithelial membrane antigen (EMA) for the differential diagnosis of liver tumors.

Methods Two hundred and thirty-five immunohistochemical sections of hepatocellular carcinoma (HCC; 120 cases), intrahepatic cholangiocarcinoma (ICC; 50 cases), combined hepatocellular and cholangiocarcinoma (CHC; 17 cases), metastatic adenocarcinoma (20 cases), and benign liver lesions (28 cases) were obtained from the Department of Pathology at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The sensitivity and specificity of the combined biomarkers GPC3/HepPar-1/Arg-1/CK19/EMA for the differential diagnosis of HCC, ICC, and CHC were calculated and analyzed retrospectively.

Results The combined biomarkers $GPC3^+/CK19^-$ had the highest specificity (98.3%) for diagnosing HCC, with a sensitivity of 60.0%. The specificity of $GPC3^-/HepPar\text{-}1^-/Arg\text{-}1^-/CK19^+/EMA^+$ for diagnosing ICC was 93.0%, with a sensitivity of 76.0%. The specificity of $GPC3^+/HepPar\text{-}1^+/Arg\text{-}1^+/CK19^+/EMA^+$ for diagnosing CHC was 95.9%, with a sensitivity of 52.9%.

Conclusion The combined biomarkers GPC3/HepPar-1/Arg-1/CK19/EMA greatly improved the specificity of liver tumor diagnosis. We believe that clinical pathological work could improve the original determination of liver nodules.

Received: 16 April 2019
Revised: 30 June 2019
Accepted: 20 July 2019

Key words: hepatocellular carcinoma (HCC); intrahepatic cholangiocarcinoma (ICC); combined hepatocellular and cholangiocarcinoma (CHC); immunohistochemistry

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer-related deaths worldwide [1]. The current gold standard for HCC diagnosis is pathological examination; however, some complicated cases can be difficult to determine, such as patients with intrahepatic cholangiocarcinoma (ICC) or combined hepatocellular and cholangiocarcinoma (CHC) [2–3]. Various immunohistochemical biomarkers have played an increasingly important role in assisting pathological diagnosis of cellular origin [4–5].

Glypican-3 (GPC3) is an important member of the glypican family that is attached to the cell membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor. GPC3 was first reported by Hsu *et al* in 1997 [6], with its protein levels in cancerous and normal liver tissues confirmed by a subsequent study [7–9]. GPC3 has a close interaction

✉ Correspondence to: Xiaohua Zhu. Email: evazhu@vip.sina.com
* Supported by grants from National Natural Science Foundation of China (NSFC; No. 81271600, 81671718 and 81873903), the Natural Science Foundation of Hubei Province in China (No. 2016CFB687) and the Clinical Foundation of Tongji Hospital (No. 2015C013).

with the Wnt, Yap, and FGF signaling pathways, which are thought to promote HCC formation and progression [10–13]. Hepatocyte paraffin antigen-1 (HepPar-1) is a surface antigen from hepatic mitochondria that is highly expressed in tissues of hepatocytic origin; it has been reported that the sensitivity of HepPar-1 during HCC diagnosis could be as high as 90% [14–16]. Arginase (Arg-1) is an enzyme that can metabolize arginine into urea and ornithine. Like HepPar-1, Arg-1 has adequate diagnostic sensitivity for HCC. Studies have shown that the sensitivity of Arg-1 for HCC detection could be > 90%, higher than alpha-foetoprotein (AFP), GPC3, and HepPar-1 [14–18]. However, both Arg-1 and HepPar-1 are expressed in some benign liver lesions, thus their diagnostic specificity for HCC is inferior to GPC3 and AFP. As for ICC diagnosis, cytokeratin-19 (CK19) is expressed in various single-layer epithelial tissues and exhibits high sensitivity [19–21]. CK19 is mainly used to differentiate adenocarcinoma from HCC in intrahepatic lesions. Human epithelial membrane antigen (EMA) is a specific tumor biomarker since its protein epitope is associated with abnormal glycosylation. As a member of transmembrane glycoprotein family, EMA is expressed in various epithelial tissues, such as ICC [19, 22]; however, both CK19 and EMA exhibit inadequate specificity for ICC since their expression can also be detected in metastatic adenocarcinoma and CHC.

To combine the characteristics of each biomarker, Timek *et al* reported that Arg-1, HepPar-1, and GPC3 formed the most effective biomarker panel for distinguishing HCC from metastatic tumors [23], whilst Ryu *et al* suggested that GPC3 and CK19 could be used as first-line markers for the differential diagnosis of HCC and ICC [20]. In this study, we used a large data set to assess the utility of the GPC3/HepPar-1/Arg-1/CK19/EMA immunohistochemical panel for differentially diagnosing intrahepatic lesions, applicable for HCC, ICC, CHC, metastatic adenocarcinoma, and benign liver lesions. This is the first report to recommend the GPC3/HepPar-1/Arg-1/CK19/EMA panel for the differential diagnosis of liver tumors. We believe that the panel will facilitate pathological diagnosis in a clinical setting.

Materials and methods

Patients and tissue samples

Patients who had been simultaneously tested for the immunohistochemical biomarkers GPC3, HepPar-1, Arg-1, CK19, and EMA were selected from the Department of Pathology of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, between January 2012 and May 2015 (120 HCC cases, 50 ICC cases, 17 CHC cases, 20 metastatic adenocarcinoma cases, and 28 benign liver lesion cases). The origins of metastatic liver adenocarcinoma included pancreatic cancer (6 cases), lung cancer (6 cases), gastric cancer (3 cases), colon cancer (2 cases), rectal cancer (2 cases) and breast cancer (1 case). Benign liver lesions included inflammatory liver hyperplasia (7 cases), fibrous liver hyperplasia (9 cases), and focal nodular liver hyperplasia (12 cases). The final decision for all cases was made by macro- and micro-pathological observations, with immunohistochemical tests assisting the diagnosis. The sensitivity and specificity of each biomarker and the combined biomarkers for the diagnosis of liver tumors was calculated. Samples were acquired from the resections or biopsies of HCC, ICC, CHC, metastatic adenocarcinoma, and benign liver lesions. All tissues were routinely fixed in 10% neutral buffered formalin and paraffin, with slides independently reviewed by two pathologists.

Immunohistochemistry and interpretation

Immunohistochemistry was performed according to the standard protocol of the Department of Pathology of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Prior to immunohistochemical staining, all available slides were routinely subjected to hematoxylin and eosin (HE) staining to identify tissue blocks with tumor architecture. The tissue blocks were then fixed in formalin, embedded in paraffin, sectioned at a thickness of 5 μm, deparaffinized, and rehydrated. For antigen retrieval, sections were soaked in 10 mM citrate buffer in a pressure cooker for 3 min. Endogenous peroxidase activity was inhibited by treatment with 3% hydrogen peroxide for 3 min and nonspecific binding sites were blocked with 10% non-immune goat serum for 30 min. The slides were treated with the following individual primary monoclonal antibodies (against GPC3, HepPar-1, Arg-1, CK19, and EMA biomarkers) at 4 °C overnight: mouse anti-GPC3 (1:150), mouse anti-HepPar-1 (1:120), rabbit anti-Arg-1 (1:120), mouse anti-CK19 (1:150), and mouse anti-EMA (1:150). The antibodies were all purchased from ZSGB-BIO (Beijing, China) and an Envision Kit + Dual Link System HRP was used to develop the histological images. Diaminobenzidine (DAB) was used as the chromogen for immunostaining. Phosphate-buffered saline (PBS, pH 7.2) was used instead of primary antibodies as the negative control, and specific GPC3, HepPar-1, Arg-1, CK19, and EMA positive samples confirmed by western blotting were used as positive controls. Before examination under an Olympus microscopic digital camera, all slides were counterstained with hematoxylin.

To interpret the immunohistochemistry results, two pathologists independently analyzed the staining under a microscope. GPC3, HepPar-1, Arg-1, CK19, and EMA were located in the cytoplasm and membrane, cytoplasm, cytoplasm and nuclei, cytoplasm, and cytoplasm,

respectively. Histological scores were given by two methods: (1) Scoring by staining intensity (0: no signal; 1: weak; 2: moderate; and 3: marked); (2) Scoring by the percentage of immunoreactive cells (0: 0%; 1: ≤ 10%; 2: > 10%–50%; 3: 50%–75%; 4: > 75%). After multiplying the two scores, scores of > 3 was considered positive expression and scores of < 3 were considered negative expression.

Statistical analysis

Calculations were performed using the SPSS 17.0 software package. Differences between the rates of biomarker positivity in different liver lesions were analyzed using chi-square tests. Values of $P < 0.05$ were considered significant.

Results

This retrospective study analyzed the ability of the GPC3/HepPar-1/Arg-1/CK19/EMA panel of immunohistochemical biomarkers to differentially diagnose liver tumors. The single biomarkers with the highest sensitivity and specificity for HCC diagnosis were Arg-1 (90.0%) and GPC3 (79.1%), respectively (Table 1). The single biomarkers with the highest sensitivity and specificity for ICC diagnosis were CK19 (98.0%) and EMA (64.9%), respectively (Table 2). The combined biomarkers $GPC3^+/CK19^-$ had the highest specificity (98.3%) for HCC diagnosis, with a sensitivity of 60.0% (Table 3). The diagnostic efficiency of $GPC3^+/EMA^-$ (specificity: 97.4%; sensitivity: 60.8%) for HCC was very similar to that of $GPC3^+/CK19^-$. The specificity of $GPC3^-/HepPar\text{-}1^-/Arg\text{-}1^-/CK19^+/EMA^+$ for ICC diagnosis was 93.0%, with a sensitivity of 76.0% (Table 4). The specificity of $GPC3^+/HepPar\text{-}1^+/Arg\text{-}1^+/CK19^+/EMA^+$ for CHC diagnosis was 95.9%, with a sensitivity of 52.9% (Table 5).

The staining sites of GPC3, HepPar-1, Arg-1, CK19, and EMA were the cytoplasm and membrane, cytoplasm, cytoplasm and nuclei, cytoplasm, and cytoplasm, respectively. The representative staining pattern of the immunohistochemical biomarkers in different liver tumors was as follows: 1. GPC3, HepPar-1, and Arg-1 were highly expressed in HCC, whilst CK19 and EMA were almost unidentifiable (Fig. 1); 2. CK19 and EMA were highly expressed in ICC whilst GPC3, HepPar-1 and Arg-1 expression was relatively low (Fig. 2), with the staining features almost the same in metastatic adenocarcinoma (Fig. 3); 3. GPC3, HepPar-1, Arg-1, CK19, and EMA were all highly expressed in CHC (Fig. 4); and 4. HepPar-1 and Arg-1 were positively expressed in benign liver lesions, whilst GPC3, CK19, and EMA were barely detectable (Fig. 5).

Discussion

Liver tumors pose a serious problem for human health, with accurate diagnosis and early intervention critical for extending survival time and improving quality of life [24–26]. However, for some complicated clinical cases it can be difficult to differentiate between liver tumors; thus, treatment can be seriously hampered due to inaccurate or delayed diagnosis. AFP is a traditional biomarker for HCC diagnosis, with AFP levels being an important indicator for HCC [14, 27–28]; however, a large number of HCC patients are AFP-negative [14, 28]. It has been reported that AFP immunoreactivity was only detected in 40 of 78 (51.3%) HCC cases by immunohistochemistry [14]. Multiple biomarkers could improve the determination of disease origin and reduce the rate of misdiagnosis and

Table 1 Expression level of GPC3, HepPar-1, and Arg-1 in different liver lesions

	HCC (n = 120)	ICC (n = 50)	P^1	CHC (n = 17)	P^2	Metastatic adenocarcinoma (n = 20)	P^3	Benign liver lesions (n = 28)	P^4	Sensitivity (%)	Specificity (%)
$GPC3^+$	95	6	0.000	14	0.760	2	0.000	2	0.000	79.2	79.1
$HepPar\text{-}1^+$	96	3	0.000	12	0.374	3	0.000	23	0.797	80.0	64.3
$Arg\text{-}1^+$	108	4	0.000	14	0.345	0	NA	25	0.910	90.0	62.6

P^1, P^2, P^3 and P^4 represented difference comparison of biomarkers' positive rates between HCC and ICC, CHC, metastatic adenocarcinoma, benign liver lesions respectively. NA: Not available. The sample cases were 0, and process can't be conducted.

Table 2 Expression level of CK19 and EMA in different liver lesions

	ICC (n = 50)	HCC (n = 120)	P^1	CHC (n = 17)	P^2	Metastatic adenocarcinoma (n = 20)	P^3	Benign liver lesions (n = 28)	P^4	Sensitivity (%)	Specificity (%)
$CK19^+$	49	26	0.000	17	NA	19	0.496	8	0.000	98.0	62.2
EMA^+	48	27	0.000	16	0.746	18	0.329	4	0.000	96.0	64.9

P^1, P^2, P^3 and P^4 represented difference comparison of biomarkers' positive rates between ICC and HCC, CHC, metastatic adenocarcinoma, benign liver lesions respectively. NA: Not available. The sample cases were 0, and process can't be conducted.

Table 3 Combined biomarkers for diagnosis of HCC (*n*, %)

	HCC (*n* = 120)	ICC (*n* = 50)	CHC (*n* = 17)	Metastatic adenocarcinoma (*n* = 20)	Benign liver lesions (*n* = 28)	Sensitivity (%)	Specificity (%)
Two							
$GPC3^+/HepPar\text{-}1^+$	76	2	10	0	2	63.3	87.8
$GPC3^+/Arg\text{-}1^+$	86	2	12	0	2	71.7	86.1
$HepPar\text{-}1^+/Arg\text{-}1^+$	93	1	12	0	21	80.9	70.4
$GPC3^+/CK19^-$	72	0	0	0	2	60.0	98.3
$HepPar\text{-}1^+/CK19^-$	79	0	0	0	18	65.8	84.3
$Arg\text{-}1^+/CK19^-$	86	0	0	0	18	71.7	84.3
$GPC3^+/EMA^-$	73	0	1	0	2	60.8	97.4
$HepPar\text{-}1^+/EMA^-$	77	0	1	0	22	64.2	80.0
$Arg\text{-}1^+/EMA^-$	87	0	1	0	22	72.5	80.0
Three							
$GPC3^+/HepPar\text{-}1^+/Arg\text{-}1^+$	76	1	10	0	2	63.3	88.7
$GPC3^+/HepPar\text{-}1^+/CK19^-$	62	0	0	0	2	51.7	98.3
$GPC3^+/Arg\text{-}1^+/CK19^-$	67	0	0	0	2	55.8	98.3
$HepPar\text{-}1^+/Arg\text{-}1^+/CK19^-$	77	0	0	0	16	64.2	86.1
$GPC3^+/HepPar\text{-}1^+/EMA^-$	59	0	1	0	2	49.2	97.4
$GPC3^+/Arg\text{-}1^+/EMA^-$	68	0	1	0	2	56.7	97.4
$HepPar\text{-}1^+/Arg\text{-}1^+/EMA^-$	76	0	1	0	20	63.3	81.7
$GPC3^+/CK19^-/EMA^-$	60	0	0	0	2	50.0	98.3
$HepPar\text{-}1^+/CK19^-/EMA^-$	69	0	0	0	17	57.5	85.2
$Arg\text{-}1^+/CK19^-/EMA^-$	75	0	0	0	18	62.5	84.3
Four							
$GPC3^+/HepPar\text{-}1^+/CK19^-/EMA^-$	52	0	0	0	2	43.3	98.3
$GPC3^+/Arg\text{-}1^+/CK19^-/EMA^-$	57	0	0	0	2	47.5	98.3
$HepPar\text{-}1^+/Arg\text{-}1^+/CK19^-/EMA^-$	67	0	0	0	16	55.8	86.1
$GPC3^+/HepPar\text{-}1^+/Arg\text{-}1^+/CK19^-$	60	0	0	0	2	50.0	98.3
$GPC3^+/HepPar\text{-}1^+/Arg\text{-}1^+/EMA^-$	56	0	1	0	2	46.7	97.4
Five							
$GPC3^+/HepPar\text{-}1^+/Arg\text{-}1^+/CK19^-/EMA^-$	51	0	0	0	2	42.5	98.3

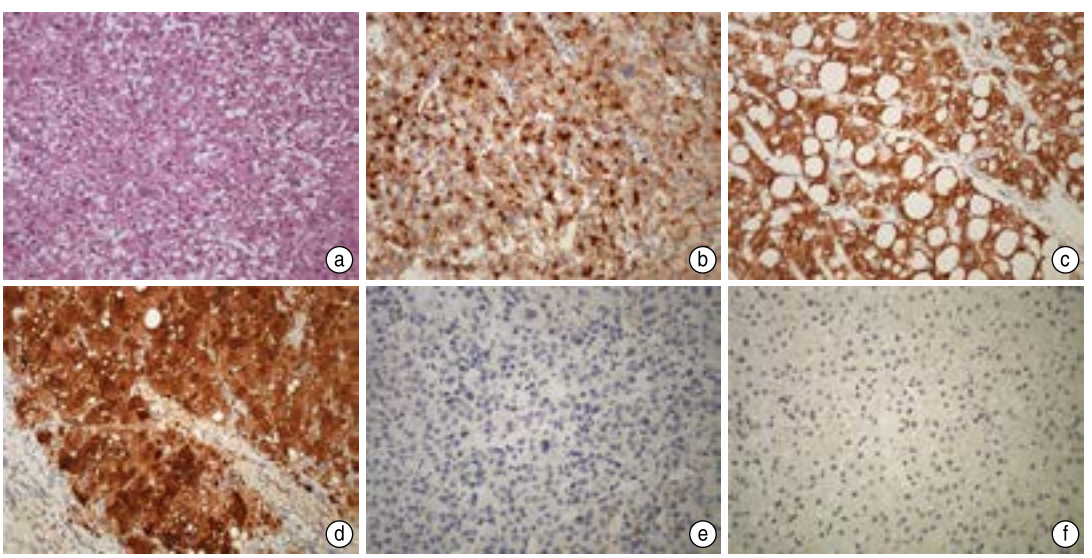

Fig. 1 HE staining of HCC and expression level of different biomarkers in HCC. (a) HE staining of HCC. (b) GPC3 was highly expressed in HCC, and the staining sites were cytoplasm and membrane. (c) HepPar-1 was highly expressed in HCC, and the staining site was cytoplasm. (d) Arg-1 was highly expressed in HCC, and the staining sites were cytoplasm and nuclei. CK19 (e) and EMA (f) were almost not presented in HCC. (× 200)

Table 4 Combined biomarkers for diagnosis of ICC (*n*, %)

	ICC (*n* = 50)	HCC (*n* = 120)	CHC (*n* = 17)	Metastatic adenocarcinoma (*n* = 20)	Benign liver lesions (*n* = 28)	Sensitivity (%)	Specificity (%)
Two							
$CK19^+/EMA^+$	47	12	16	17	4	94.0	73.5
$GPC3^-/CK19^+$	43	3	3	18	8	86.0	82.7
$HepPar\text{-}1^-/CK19^+$	46	4	5	16	3	92.0	84.9
$Arg\text{-}1^-/CK19^+$	45	4	3	19	1	90.0	85.4
$GPC3^-/EMA^+$	42	5	3	16	4	84.0	84.9
$HepPar\text{-}1^-/EMA^+$	45	8	5	15	3	90.0	85.2
$Arg\text{-}1^-/EMA^+$	44	6	3	18	1	88.0	84.9
Three							
$GPC3^-/HepPar\text{-}1^-/CK19^+$	42	1	1	14	3	84.0	89.7
$GPC3^-/Arg\text{-}1^-/CK19^+$	41	0	1	17	0	82.0	90.3
$HepPar\text{-}1^-/Arg\text{-}1^-/CK19^+$	43	4	3	16	0	86.0	87.6
$GPC3^-/HepPar\text{-}1^-/EMA^+$	41	3	1	14	3	82.0	88.6
$GPC3^-/Arg\text{-}1^-/EMA^+$	40	2	1	16	1	80.0	89.2
$HepPar\text{-}1^-/Arg\text{-}1^-/EMA^+$	42	5	3	15	1	84.0	87.0
$GPC3^-/CK19^+/EMA^+$	41	2	3	15	4	82.0	87.0
$HepPar\text{-}1^-/CK19^+/EMA^+$	45	3	5	14	4	90.0	85.9
$Arg\text{-}1^-/CK19^+/EMA^+$	43	2	3	17	1	86.0	87.6
Four							
$GPC3^-/HepPar\text{-}1^-/CK19^+/EMA^+$	41	1	1	12	3	82.0	90.8
$GPC3^-/Arg\text{-}1^-/CK19^+/EMA^+$	40	0	1	15	0	80.0	91.4
$HepPar\text{-}1^-/Arg\text{-}1^-/CK19^+/EMA^+$	41	2	3	14	0	82.0	89.7
$GPC3^-/HepPar\text{-}1^-/Arg\text{-}1^-/CK19^+$	40	0	1	14	0	80.0	91.9
$GPC3^-/HepPar\text{-}1^-/Arg\text{-}1^-/EMA^+$	39	1	1	13	1	78.0	91.4
Five							
$GPC3^-/HepPar\text{-}1^-/Arg\text{-}1^-/CK19^+/EMA^+$	38	0	1	12	0	76.0	93.0

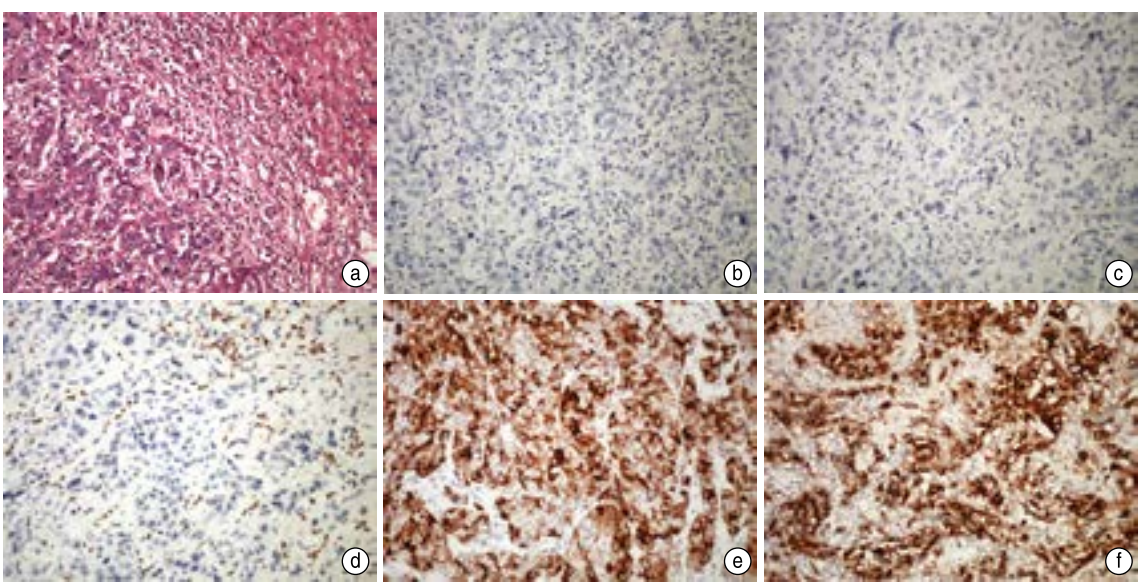

Fig. 2 HE staining of ICC and expression level of different biomarkers in ICC. (a) HE staining of ICC. GPC3 (b), HepPar-1 (c) and Arg-1 (d) were negatively expressed in ICC. CK19 (e) and EMA (f) were highly expressed in ICC, and the staining site was located at cytoplasm. (× 200)

Table 5 Combined biomarkers for diagnosis of CHC (*n*, %)

	CHC (*n* = 17)	HCC (*n* = 120)	ICC (*n* = 50)	Metastatic adenocarcinoma (*n* = 20)	Benign liver lesions (*n* = 28)	Sensitivity (%)	Specificity (%)
Two							
GPC3$^+$/CK19$^+$	14	23	6	2	0	82.4	85.8
HepPar-1$^+$/CK19$^+$	12	16	3	3	5	70.6	87.6
Arg-1$^+$/CK19$^+$	14	21	4	0	7	82.4	85.3
GPC3$^+$/EMA$^+$	13	22	6	2	0	76.5	86.2
HepPar-1$^+$/EMA$^+$	11	19	3	3	1	64.7	88.1
Arg-1$^+$/EMA$^+$	13	21	4	0	3	76.5	87.2
Three							
GPC3$^+$/HepPar-1$^+$/CK19$^+$	10	15	2	0	0	58.8	92.2
GPC3$^+$/Arg-1$^+$/CK19$^+$	12	19	2	0	0	70.6	90.4
HepPar-1$^+$/Arg-1$^+$/CK19$^+$	12	17	1	0	5	70.6	89.4
GPC3$^+$/HepPar-1$^+$/EMA$^+$	9	17	2	0	0	52.9	91.3
GPC3$^+$/Arg-1$^+$/ EMA$^+$	11	20	2	0	0	64.7	89.9
HepPar-1$^+$/Arg-1$^+$/EMA$^+$	11	18	1	0	1	64.7	90.8
GPC3$^+$/CK19$^+$/EMA$^+$	13	10	6	2	0	76.5	91.7
HepPar-1$^+$/CK19$^+$/EMA$^+$	11	9	3	3	1	64.7	92.7
Arg-1$^+$/CK19$^+$/EMA$^+$	13	10	3	0	2	76.5	93.1
Four							
GPC3$^+$/HepPar-1$^+$/CK19$^+$/EMA$^+$	9	8	2	0	0	52.9	95.4
GPC3$^+$/ Arg-1$^+$/CK19$^+$/EMA$^+$	11	8	2	0	0	64.7	95.4
Hepar-1$^+$/Arg1$^+$/CK19$^+$/EMA$^+$	11	9	1	0	1	64.7	95.0
GPC3$^+$/HepPar-1$^+$/Arg-1$^+$/CK19$^+$	10	13	1	0	0	58.8	93.6
GPC3$^+$/HepPar-1$^+$/Arg-1$^+$/EMA$^+$	9	16	1	0	0	52.9	92.2
Five							
GPC3$^+$/HepPar-1$^+$/Arg-1$^+$/CK19$^+$/EMA$^+$	9	8	1	0	0	52.9	95.9

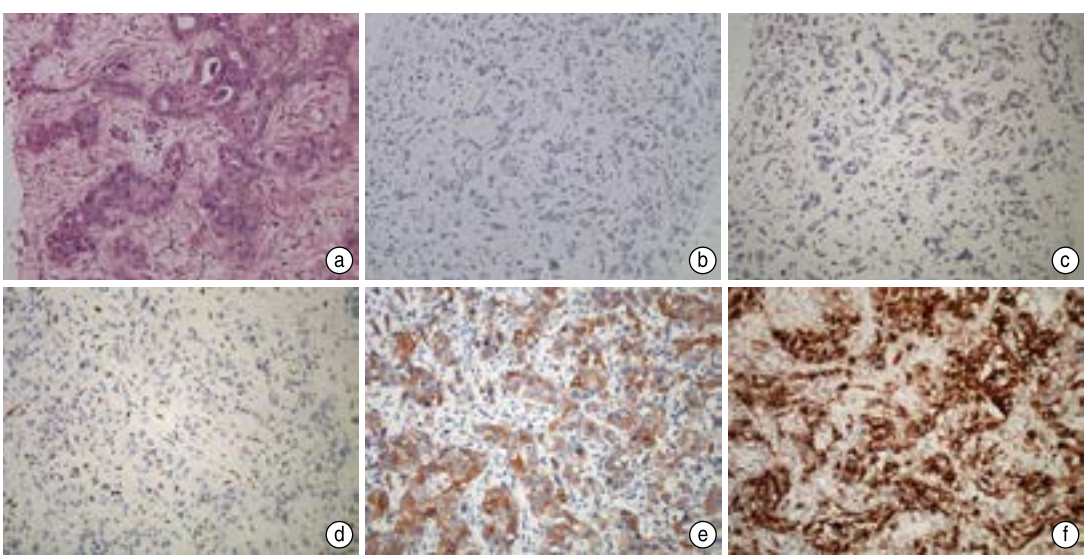

Fig. 3 HE staining of metastatic adenocarcinoma and expression level of different biomarkers in metastatic adenocarcinoma. (a) HE staining of metastatic adenocarcinoma. GPC3 (b), HepPar-1 (c) and Arg-1 (d) were negatively expressed in metastatic adenocarcinoma, while CK19 (e) and EMA (f) were highly expressed. (× 200)

missed diagnosis.

This study analyzed the usefulness of an GPC3/HepPar-1/Arg-1/CK19/EMA immunostaining panel for diagnosing and differentially diagnosing liver tumors. GPC3 is a cell surface proteoglycan that is highly expressed in early HCC but little expressed in benign

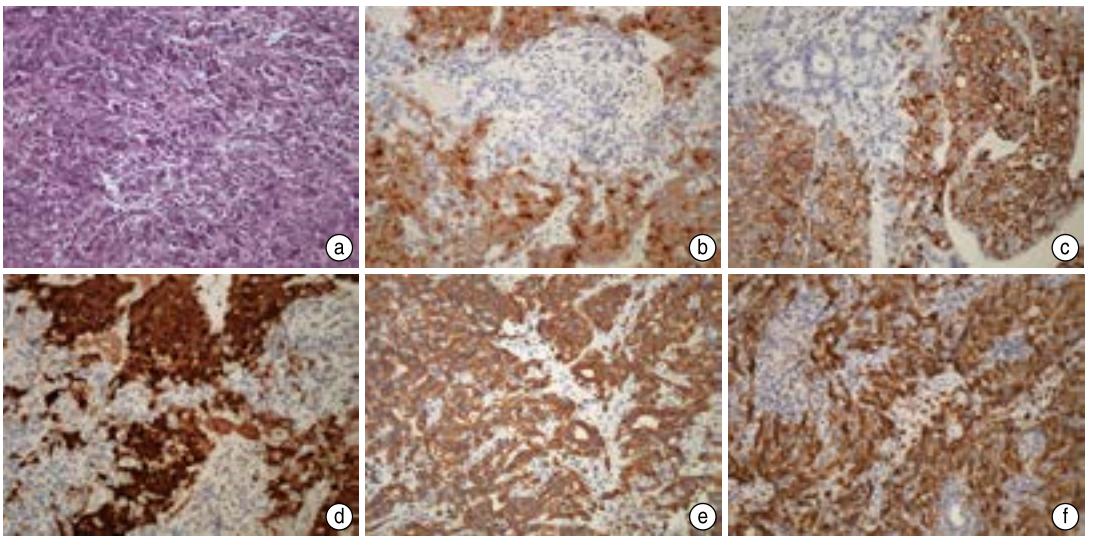

Fig. 4 HE staining of CHC and expression level of different biomarkers in CHC. (a) HE staining of CHC. GPC3 (b), HepPar-1 (c), Arg-1 (d), CK19 (e) and EMA (f) were all highly expressed in CHC. (× 200)

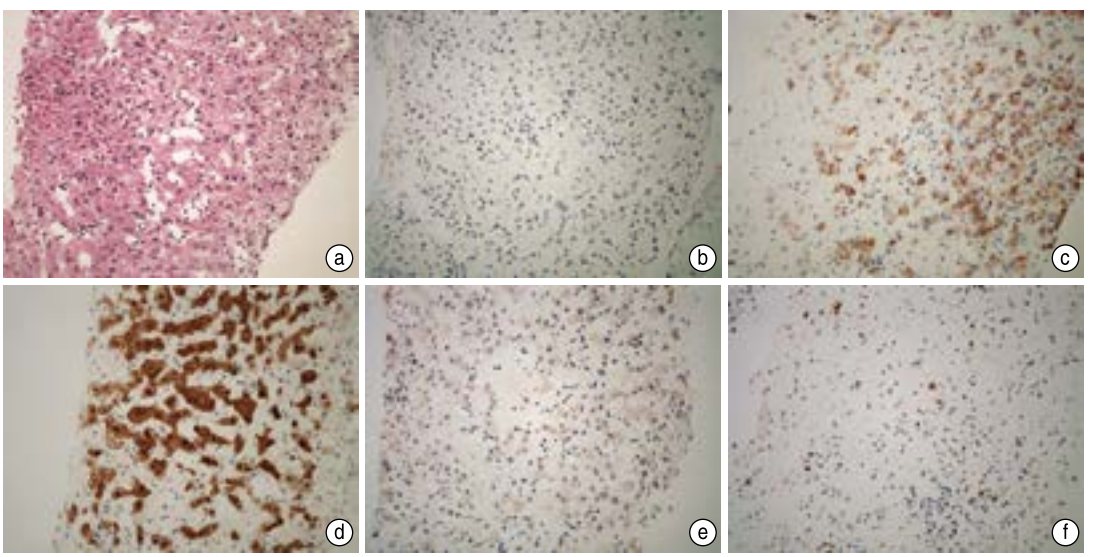

Fig. 5 HE staining of benign liver lesions and expression level of different biomarkers in benign liver lesions. (a) HE staining of benign liver lesions. HepPar-1 (c) and Arg-1 (d) were positively expressed in benign liver lesions, while GPC3 (b), CK19 (e) and EMA (f) were negatively expressed. (× 200)

liver lesions [6–9] and closely associated with tumor growth and development. High GPC3 expression levels in HCC may suggest poor differentiation, early metastasis, and poor prognosis [29]. A previous report showed that GPC3 immunostaining was positive in 78.3% (36/46) of HCCs and 72.7% (8/11) of the HCC components of CHC sections, yet negative in ICCs [30]. In our study, GPC3 immunostaining was positive in 79.2% (95/120) of HCCs and 82.4% (14/17) of CHCs, but few ICC (6/50), metastatic adenocarcinoma (2/20), and benign liver lesion (2/28) samples. Thus, GPC3 is a sensitive and specific biomarker for identifying malignant hepatic cells.

Unlike GPC3, HepPar-1 is a positive biomarker for hepatocyte differentiation that is highly expressed in both malignant and non malignant hepatic cells [31]. The rates of HepPar-1 positivity in HCC and benign liver lesions were 80.0% (96/120) and 82.1% (23/28), respectively. Due to the high level of HepPar-1 expression in CHC (70.6%, 12/17) and benign liver lesions, the specificity of HepPar-1 for HCC diagnosis was only 64.3%. Consistent with previous studies, HepPar-1 was observed in other tumor types, with 3/50 ICC cases and 3/20 metastatic

adenocarcinoma cases staining HepPar-1-postive [14, 32]. As an enzyme involved in the urea cycle, Arg-1 is a more sensitive biomarker for hepatocytes than HepPar-1 [14, 16]. The Arg-1 positivity rates in HCC and benign liver lesions were 90.0% (108/120) and 89.3% (25/28), respectively, higher than that of HepPar-1. Like HepPar-1, the specificity of Arg-1 for HCC diagnosis was only 62.6%. Our results suggest that Arg-1 was a better biomarker than HepPar-1 for distinguishing HCC from metastatic adenocarcinoma (Arg-1 was absent in all 20 metastatic liver adenocarcinoma cases) [14, 18, 23].

To identify ICC or ICC components in CHC, we performed CK19 immunostaining. CK19 is an important cytokeratin (CK) that is mainly expressed in epithelial cells, such as those in mammary gland ducts, intestinal villi, pancreatic ducts, and liver bile ducts, but not in hepatocytes [33]. It has been reported that CK19 plays a critical role in epithelial cell proliferation and differentiation. Several studies have utilized CK19 to differentiate HCC from ICC, with the CK19 positivity rate for ICC almost 90.0% [19–20, 33]. In our study, CK19 immunoreactivity was observed in 49/50 ICC cases (98.0%). EMA, another ICC-positive biomarker was also selected and analyzed. The EMA positivity rate in ICC was 96.0% (48/50 cases), with some HCC cases (27/127) also staining EMA-positive, as reported previously [19, 22, 34]. Almost all metastatic adenocarcinomas were CK19 (19/20) and EMA-positive (18/20), with the specificity of CK19 and EMA for ICC diagnosis just 62.2% and 64.9%, respectively.

In summary, GPC3 exhibited satisfactory sensitivity and specificity for HCC diagnosis. CK19 and EMA possessed adequate sensitivity for diagnosing ICC; however, their specificities were insufficient. The combination of GPC3 and CK19 or EMA may help better differentiate HCC from CHC and ICC. For the differential diagnosis of intrahepatic lesions, the single biomarkers HepPar-1 or Arg-1 could only partially suggest that the abnormality was hepatocyte-derived; thus, combining GPC3, CK19, and EMA is necessary to determine whether the disease is HCC, CHC, or a benign liver lesion. When the biomarkers GPC3, HepPar-1, Arg-1, CK19, and EMA were combined, the specificity for HCC, ICC, and CHC diagnosis increased to 98.3%, 93.0%, and 95.9%, respectively. Based on the expression features of each biomarker for liver tumor diagnosis, GPC3 was the first choice due to its high sensitivity and specificity for HCC diagnosis. The sensitivities of HepPar-1 and Arg-1 were both adequate for detecting HCC; therefore, we recommend that HepPar-1 or Arg-1 be added subjectively, with the recommended index for Arg-1 higher than that of HepPar-1 for identifying intrahepatic hepatocytes. CK19 and EMA both exhibited high sensitivity for ICC diagnosis. We recommend that CK19 and EMA be selected alternatively, with the chosen one combined with GPC3, HepPar-1, and Arg-1 to effectively differentiate HCC from ICC and CHC. There was one limitation of the GPC3/HepPar-1/Arg-1/CK19/EMA panel, since ICC and metastatic adenocarcinoma could not be differentiated well even when all biomarkers were utilized. The problem could be solved by integrating macro- and micro-pathological observations, the patients' clinical history, and other specific biomarker immunostaining.

In conclusion, we showed that the GPC3/HepPar-1/Arg-1/CK19/EMA panel of immunohistochemical biomarkers could support the diagnosis and differential diagnosis of most liver tumors, bring convenience to pathologists, and improve the accurate diagnosis and timely treatment of patients.

Conflicts of interest

The authors indicate no potential conflicts of interest.

References

1. Bosetti C, Turati F, La Vecchia C. Hepatocellular carcinoma epidemiology. Best Pract Res Clin Gastroenterol, 2014, 28: 753–770.
2. Cong WM, Bu H, Chen J, *et al*. Practice guidelines for the pathological diagnosis of primary liver cancer: 2015 update. World J Gastroenterol, 2016, 22: 9279–9287.
3. Marquardt JU, Galle PR, Teufel A. Molecular diagnosis and therapy of hepatocellular carcinoma (HCC): an emerging field for advanced technologies. J Hepatol, 2012, 56: 267–275.
4. Idikio HA. Immunohistochemistry in diagnostic surgical pathology: contributions of protein life-cycle, use of evidence-based methods and data normalization on interpretation of immunohistochemical stains. Int J Clin Exp Pathol, 2009, 3: 169–176.
5. McCourt CM, Boyle D, James J, *et al*. Immunohistochemistry in the era of personalised medicine. J Clin Pathol, 2013, 66: 58–61.
6. Hsu HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. Cancer Res, 1997, 57: 5179–5184.
7. Wang F, Jing X, Wang T, *et al*. Differential diagnostic value of GPC3-CD34 combined staining in small liver nodules with diameter less than 3 cm. Am J Clin Pathol, 2012, 137: 937–945.
8. Tremosini S, Forner A, Boix L, *et al*. Prospective validation of an immunohistochemical panel (glypican 3, heat shock protein 70 and glutamine synthetase) in liver biopsies for diagnosis of very early hepatocellular carcinoma. Gut, 2012, 61: 1481–1487.
9. Zhu D, Qin Y, Wang J, *et al*. Novel glypican-3-binding peptide for *in vivo* hepatocellular carcinoma fluorescent imaging. Bioconjug Chem, 2016, 27: 831–839.
10. Capurro M, Martin T, Shi W, *et al*. Glypican-3 binds to frizzled and plays a direct role in the stimulation of canonical Wnt signaling. J Cell Sci, 2014, 127: 1565–1575.
11. Gao W, Kim H, Feng M, *et al*. Inactivation of Wnt signaling by a human antibody that recognizes the heparan sulfate chains of glypican-3 for liver cancer therapy. Hepatology, 2014, 60: 576–587.
12. Fico A, Maina F, Dono R. Fine-tuning of cell signaling by glypicans. Cell Mol Life Sci, 2011, 68: 923–929.

13. Miao HL, Pan ZJ, Lei CJ, *et al*. Knockdown of GPC3 inhibits the proliferation of Huh7 hepatocellular carcinoma cells through down-regulation of YAP. J Cell Biochem, 2013, 114: 625–631.
14. Sang W, Zhang W, Cui W, *et al*. Arginase-1 is a more sensitive marker than HepPar-1 and AFP in differential diagnosis of hepatocellular carcinoma from nonhepatocellular carcinoma. Tumour Biol, 2015, 36: 3881–3886.
15. Fujiwara M, Kwok S, Yano H, *et al*. Arginase-1 is a more sensitive marker of hepatic differentiation than HepPar-1 and glypican-3 in fine-needle aspiration biopsies. Cancer Cytopathol, 2012, 120: 230–237.
16. Nguyen T, Phillips D, Jain D, *et al*. Comparison of 5 immunohistochemical markers of hepatocellular differentiation for the diagnosis of hepatocellular carcinoma. Arch Pathol Lab Med, 2015, 139: 1028–1034.
17. Chandan VS, Shah SS, Torbenson MS, *et al*. Arginase-1 is frequently positive in hepatoid adenocarcinomas. Hum Pathol, 2016, 55: 11–16.
18. Radwan NA, Ahmed NS. The diagnostic value of arginase-1 immunostaining in differentiating hepatocellular carcinoma from metastatic carcinoma and cholangiocarcinoma as compared to HepPar-1. Diagn Pathol, 2012, 7: 149.
19. Maeno S, Kondo F, Sano K, *et al*. Morphometric and immunohistochemical study of cholangiolocellular carcinoma: comparison with non-neoplastic cholangiole, interlobular duct and septal duct. J Hepatobiliary Pancreat Sci, 2012, 19: 289–296.
20. Ryu HS, Lee K, Shin E, *et al*. Comparative analysis of immunohistochemical markers for differential diagnosis of hepatocelluar carcinoma and cholangiocarcinoma. Tumori, 2012, 98: 478–484.
21. Qin J, Wang M, Qin J, *et al*. High promoter activity of cytokeratin-19 gene in cholangiocarcinoma. Mol Clin Oncol, 2018, 9: 467–471.
22. Balitzer D, Joseph NM, Ferrell L, *et al*. Immunohistochemical and molecular features of cholangiolocellular carcinoma are similar to well-differentiated intrahepatic cholangiocarcinoma. Mod Pathol, 2019, Jun 11. doi: 10.1038/s41379-019-0290-0. [Epub ahead of print].
23. Timek DT, Shi J, Liu H, *et al*. Arginase-1, HepPar-1, and Glypican-3 are the most effective panel of markers in distinguishing hepatocellular carcinoma from metastatic tumor on fine-needle aspiration specimens. Am J Clin Pathol, 2012, 138: 203–210.
24. Wong KF, Xu Z, Chen J, *et al*. Circulating markers for prognosis of hepatocellular carcinoma. Expert Opin Med Diagn, 2013, 7: 319–329.
25. Wang Z, Yang G, Nie P, *et al*. Dynamical observation on biological progression of VX2 liver tumors to identify the optimal time for intervention in animal models. PLoS One, 2013, 8: e74327.
26. Xie S, Tang LG, Cai X, *et al*. Might liver transplantation recipients with primary hepatocellular carcinoma benefit from GVT effect of aGVHD? Chinese-German J Clin Oncol, 2014, 13: 535–538.
27. Wang L, Yao M, Yao Y, *et al*. Diagnostic and monitoring values of circulating AFP-mRNA and HS-GGT for hepatocellular carcinoma. Chinese-German J Clin Oncol, 2014, 13: 457–463.
28. Sauzay C, Petit A, Bourgeois AM, *et al*. Alpha-foetoprotein (AFP): A multi-purpose marker in hepatocellular carcinoma. Clin Chim Acta, 2016, 463: 39–44.
29. Shirakawa H, Suzuki H, Shimomura M, *et al*. Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci, 2009, 100: 1403–1407.
30. Shirakawa H, Kuronuma T, Nishimura Y, *et al*. Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. Int J Oncol, 2009, 34: 649–656.
31. McKnight R, Nassar A, Cohen C, *et al*. Arginase-1: a novel immunohistochemical marker of hepatocellular differentiation in fine needle aspiration cytology. Cancer Cytopathol, 2012, 120: 223–229.
32. Fan Z, van de Rijn M, Montgomery K, *et al*. Hep par 1 antibody stain for the differential diagnosis of hepatocellular carcinoma: 676 tumors tested using tissue microarrays and conventional tissue sections. Mod Pathol, 2003, 16: 137–144.
33. Liu LZ, Yang LX, Zheng BH, *et al*. Ck7/CK19 index: A potential prognostic factor for postoperative intrahepatic cholangiocarcinoma patients. J Surg Oncol, 2018, 117: 1531–1539.
34. Kim GJ, Rhee H, Yoo JE, *et al*. Increased expression of CCN2, epithelial membrane antigen, and fibroblast activation protein in hepatocellular carcinoma with fibrous stroma showing aggressive behavior. PLoS One, 2014, 9: e105094.

DOI 10.1007/s10330-019-0351-1
Cite this article as: Zhu DL, Kuang D, Zou SJ, *et al*. Immunohistochemical panel of glypican-3, hepatocyte paraffin antigen-1, arginase-1, cytokeratin-19, and human epithelial membrane antigen for the differential diagnosis of liver tumors. Oncol Transl Med, 2019, 5: 153–161.