Original Article

Diagnostic value of glypican-3, arginase-1 and hepatocyte paraffin antigen -1 in differentiating hepatocellular carcinoma from intrahepatic cholangiocarcinoma

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Background: The aim of the present study was to investigate the diagnostic value of glypican-3 (GPC3), arginase-1 (Arg-1), and hepatocyte paraffin antigen 1 (HepPar-1) in differentiating hepatocellular carcinoma (HCC) from intrahepatic cholangiocarcinoma (ICC).

Methods: The expression of GPC3, HepPar-1 and Arg-1 were measured by immunohistochemistry in 47 cases of HCC, 29 cases of ICC and their paracancerous tissues.

Results: A high expression of GPC3, Arg-1 and HepPar-1 was observed in HCC tissues (68.09%, 76.60% and 78.72%, respectively; P>0.0125) while it was lower in ICC tissues (6.90%, 6.90% and 13.79%, respectively; P>0.0125). With regard to specificity, GPC3 performed better than Arg-1 and HepPar-1 (97.37% *vs.* 1.32% and 2.63%, respectively; P<0.05). The positive rate in poorly differentiated HCC for either GPC3, Arg-1 or HepPar-1 was lower than that in well- and moderately differentiated HCC. The majority of positive samples for GPC3 and Arg-1 were grade 2+ in well-, moderately- or poorly-differentiated HCC. Combined detection of GPC3, Arg-1 and HepPar-1 could increase the sensitivity up to 89.36% and the specificity to 100.00% , comparing with any single biomarker (P<0.05).

Conclusions: GPC3, Arg-1 and HepPar-1 were all useful biomarkers in differentiating HCC from ICC. The combination models could improve the diagnosis value of HCC and help differentiating HCC from ICC.

Keywords: Hepatocellular carcinoma (HCC); glypican-3 (GPC3); hepatocyte paraffin antigen 1 (HepPar-1); arginase-1 (Arg-1); biomarker

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, ranking fifth in frequency and the third leading cause of cancer-related mortality (1-3). The incidence of HCC has significantly increased in China; it accounts for 50% of the annual incidence of tumors and is the second cause of cancer-related deaths (4-6). The distinction of HCC from intrahepatic cholangiocarcinoma (ICC) and other types of metastatic adenocarcinoma to the liver is a challenge for its diagnosis and treatment (7).

Table 1 The differentiation of the specimens

Cancer	Well-differentiated	Moderately differentiated	Poorly differentiated	Total
HCC	19	20	8	47
ICC	4	17	8	29

HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

Some traditionally recognized hepatocyte-specific markers, including hepatocyte paraffin antigen 1 (HepPar-1), Golgi protein 73 (GP73), CD34, CD31, CD10 and α-fetoprotein, are considered to be insufficient for the diagnosis of HCC, particularly in cases of poorly differentiated HCC and metastatic tumors (8-11). Over the last two decades, it has become urgent to discover and evaluate new biomarkers for better initial and differential diagnosis of HCC. Several new biomarkers which highly expressed in HCC, including glypican-3 (GPC3) (12-16) and arginase-1 (Arg-1) (17-19), have been recognized as useful diagnostic biomarkers in differentiating HCC from metastatic tumors or ICC. However, the reported results were somehow inconsistent, and their value has not yet been confirmed.

In the present study, the diagnostic value of GPC3, Arg-1 and HepPar-1 in differentiating HCC from ICC was investigated by immunohistochemical staining, with a self-produced monoclonal antibody against GPC3 (20). Different combination models for these three biomarkers were also evaluated in terms of their ability to diagnose HCC and differentiated HCC from ICC.

Methods

Tissue specimens

This retrospective study included 47 cases of HCC (37 men and 10 women aged 28–82 years) and 29 cases of ICC (22 men and 7 women aged 40–78 years). The differentiation stages of the specimens are shown in *Table 1*. All cases were retrieved from the archives of the First Affiliated Hospital of Zhejiang University between 2012 and 2014. The clinical history, pathology reports and hematoxylin and eosin-stained slides for all cases were reviewed to confirm the diagnosis, according to the World Health Organization criteria (2008 edition) and the International Consensus Group for Hepatocellular Neoplasia in 2009 (21). Follow-up information of both recurrence and survival in HCC patients was collected as much as possible for nearly three-years. The study was approved by the Ethical Committee of the First Affiliated Hospital of Zhejiang University (REC number: EC-2015-82). The specimens were obtained with the consent of the patients and signed consent forms are kept in the medical Records Library.

Reagents and immunohistochemistry

Four-micron-thick sections of the formalin-fixed, paraffin-embedded tissue blocks of all the studied cases (including their paired paracancerous tissues) were prepared for immunohistochemistry targeted to GPC3, Arg-1 and HepPar-1. Immunohistochemistry was performed using the immunoperoxidase method. Positive control and negative control were set. In brief, sections were deparaffinized with xylene and rehydrated through a series of ethanol solutions. Heat-induced antigen retrieval was conducted in 0.1 mol/L citrate buffer (pH 6.0) in a microwave for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 15 min. Pretreated sections were incubated with primary mouse monoclonal antibody against GPC3 (clone 7D11; 1:100 dilution; Darui Biotechnology, Guangzhou, China), HepPar-1 (clone OCH1E5; 1:200 dilution; DakoCytomation, Carpinteria, CA, USA) and Arg-1 (HPA003595; 1:200 dilution, Merck KGaA, Darmstadt, Germany) overnight at 4 ℃. The reaction was detected with EnVision™ + Dual Link System-HRP (DAB) kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Sections were counter stained with hematoxylin for 15 sec before being checked under a microscope. For the negative control, phosphate-buffered saline was substituted for the primary antibody.

Scoring of immunostaining

Semi-quantitative analysis was used to assess staining intensity and percentages of the cells. A four-tiered scale was introduced based on the intensity and the total percentage of positive cells as follows: Negative (no staining/weak staining and ≤10% stained), 1+ (weak staining but >10% stained, or dark staining with 5–10% stained), 2+ (moderate staining, and 10–50% stained), and 3+ (dark staining, and >50% stained). The Scoring of immunostaining work was done by two researchers independently. The inconsistent results were re-scored again.

Statistical analysis

The positive rate, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of Arg-

Table 2 The expression results of GPC3, Arg-1, and HepPar-1 in different tissues

Cancer kind	Total	GPC3		Arg-1		HepPar-1	
		(–)	(+)	(–)	(+)	(–)	(+)
HCC	47	15 (31.91%)	32 (68.09%)	11 (23.40%)	36 (76.60%)	10 (21.28%)	37 (78.72%)
Well-differentiated	19	5 (26.32%)	14 (73.68%)	5 (26.32%)	14 (73.68%)	4 (21.05%)	15 (78.95%)
Moderately differentiated	20	7 (35.00%)	13 (65.00%)	3 (15.00%)	17 (85.00%)	3 (15.00%)	17 (85.00%)
Poorly differentiated	8	3 (37.50%)	5 (62.50%)	3 (37.50%)	5 (62.50%)	3 (37.50%)	5 (62.50%)
ICC	29	27 (93.10%)	2 (6.90%)	27 (93.10%)	2 (6.90%)	25 (86.21%)	4 (13.79%)
Well-differentiated	4	4 (100.00%)	0 (0.00%)	4 (100.00%)	0 (0.00%)	3 (75.00%)	1 (25.00%)
Moderately differentiated	17	16 (94.12%)	1† (5.88%)	15 (88.24%)	2† (11.76%)	15 (88.24%)	2† (11.76%)
Poorly differentiated	8	7 (87.50%)	1 (12.50%)	8 (100.00%)	0 (0.00%)	7 (87.50%)	1 (12.50%)
Paracancerous tissue	76	74 (97.37%)	2 (2.63%)	1 (1.32%)	75 (98.68%)	2 (2.63%)	74 (97.37%)

†, represented the sample BC99. GPC3, glypican-3; Arg-1, arginase-1; HepPar-1, hepatocyte paraffin antigen 1; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

1, HepPar-1 and GPC3 were analyzed for significance. Statistical analysis was performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). Paired crosstabs were sorted out and McNemar's test was done for the group comparison of Positive rate, sensitivity, or specificity. P<0.05 was set for statistically significant for each crosstab. When the compared groups (k) are three or more than three, the formula $\alpha'=\frac{\alpha}{\frac{k(k-1)}{2}+1}$ was used for the check level cutting. As for the comparison of the expression rate for each biomarker in different sample groups, Pearson Chi-square test (or Fisher's Exact Test) was chosen. P<0.05 was set for statistically significant.

Results

GPC3, Arg-1 and HepPar-1 expression rate in different tissues

The immunostaining results (see *Table 2*) showed that GPC3, Arg-1 and HepPar-1 all had a higher expression rate in HCC tissues (68.09%, 76.60% and 78.72%, respectively) and lower in ICC tissues (6.90%, 6.90% and 13.79%, respectively) (P<0.05). There were no obvious difference of the expression rates between these three biomarkers both in HCC and in ICC (P>0.0125). No obvious difference in the expression distribution was observed between these three biomarkers in the different differentiated HCC. Therefore, the positive rate in poorly differentiated HCC for either GPC3 (62.50% *vs.* 73.68% and 65.00%), Arg-1 (62.50% *vs.* 73.68% and 85.00%) or HepPar-1 (62.50% *vs.* 78.95% and 85.00%), appeared to be slightly lower than that in well- and moderately differentiated HCC. With regard to specificity, GPC3 performed better than Arg-1 and HepPar-1 (97.37% *vs.* 1.32% and 2.63%), while there was no difference in specificity between Arg-1 and HepPar-1 (see *Table 3* and *Figure 1*). Difference was observed among the expression rate of GPC3, Arg-1 and HepPar-1 in HCC, ICC and paracancerous tissues, respectively (P<0.05). Furthermore, sample BC99, which was originally classified as ICC, showed positive results for GPC3, Arg-1 and HepPar-1 and was re-evaluated as HCC.

GPC3, Arg-1 and HepPar-1 expression levels in different tissues

According to the scoring system, the expression level results of GPC3, Arg-1 and HepPar-1 are shown in *Table 4*. With regard to GPC3 and Arg-1, the majority of positive samples were grade 2+ in well- (52.63, 42.11%), moderately- (50.00, 60.00%) or poorly differentiated HCC (37.50, 62.50%), while the distribution of HepPar-1 was found slightly different (see *Figure 2A,B,C*). On the other hand, the expression intensity of HepPar-1 in poorly differentiated HCC seemed much higher than that of GPC3 and Arg-1 (40.00% *vs.* 0.00% and 0.00% for grade 3+).

Diagnostic value of GPC3, Arg-1 and HepPar-1 in differentiating HCC from ICC

As shown in *Table 4*, since Arg-1 and HepPar-1 were

Table 3 The diagnostic value of different biomarkers and their combination for HCC and ICC

Biomarker (s)	HCC (n=47), paracancerous tissue (n=47)				ICC (n=29)	
	Sensitivity	Specificity	PPV	NPV	(+)	(–)
GPC3	68.09% (32/47)	100.00% (47/47)	100.00% (32/32)	75.81% (47/62)	2 (6.90%)	27 (93.10%)
Arg-1	76.60% (36/47)	2.13% (1/47)	43.90% (36/82)	8.33% (1/12)	2 (6.90%)	27 (93.10%)
HepPar-1	78.72% (37/47)	0.00% (0/47)	44.05% (37/84)	0.00% (0/10)	4 (13.79%)	25 (86.21%)
GPC3 + Arg-1	87.23% (41/47)	100.00% (47/47)	100.00% (41/41)	88.68% (47/53)	1[§] (3.45%)	28 (96.55%)
GPC3 + HepPar-1	87.23% (41/47)	100.00% (47/47)	100.00% (41/41)	88.68% (47/53)	1[§] (3.45%)	28 (96.55%)
Arg-1 + HepPar-1	85.11% (40/47)	2.13% (1/47)	46.51% (40/86)	12.50% (1/8)	1[§] (3.45%)	28 (96.55%)
GPC3 + Arg-1 + HepPar-1	89.36% (42/47)	100.00% (47/47)	100.00% (42/42)	90.38% (47/52)	1[§] (3.45%)	28 (96.55%)

[§], represented the sample BC99. GPC3, glypican-3; Arg-1, arginase-1; HepPar-1, hepatocyte paraffin antigen 1; PPV, positive predictive value; NPV, negative predictive value; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

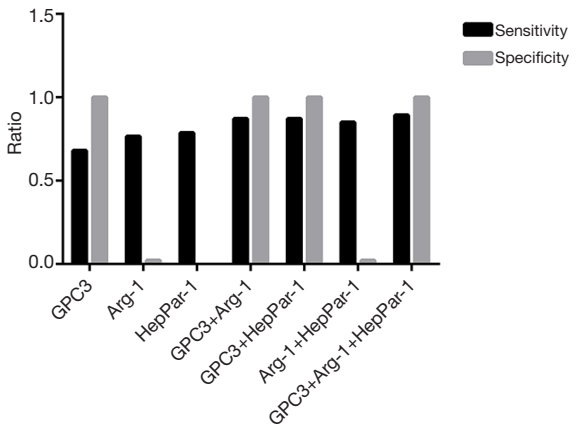

Figure 1 The sensitivity and specificity for different biomarkers and their combination in HCC. HCC, hepatocellular carcinoma.

recognized as hepatocyte-specific markers and not only specific for HCC, their PPVs lower than that of GPC3 in diagnosing HCC (43.90% and 44.05% *vs.* 100.00%, respectively; P<0.05). GPC3 had the best specificity for HCC (100%), along with a reasonable sensitivity (68.09%). GPC3 combined with Arg-1 or HepPar-1, or Arg-1 and HepPar-1, had an improved sensitivity (P=0.04, 0.04, 0.02; <0.05, respectively) and kept the well specificity in diagnosing HCC (see *Figure 1*). Meanwhile, in poorly differentiated HCC the positive number was increased only from 5 to 6 when GPC3 was combined with Arg-1 or HepPar-1, or Arg-1 and HepPar-1, and no difference was observed when Arg-1 was combined with HepPar-1. The interesting thing was that sample BC99, which positively expressing GPC3, Arg-1 and HepPar-1, was originally classified as ICC and later re-evaluated as HCC. Furthermore, 2 samples that tested negative for Arg-1 and HepPar-1, which were originally classified as HCC, were later re-evaluated as non-HCC.

The prognosis value of GPC3 for HCC patients

Follow-up information of both recurrence and survival in HCC patients was collected for nearly three-years, and 39 of 47 have been obtained. Among them, 7 were loss to follow-up for recurrence and 13 for survival. The recurrence rate in GPC3 positive group seemed little higher than that in GPC3 negative group (13/22 *vs.* 4/10), but no statistical significance was found (P=0.3158, >0.05). The analysis of DFS and OS also showed the same results (see *Figure 3*). There are 4 patients (4/18) reached the point of death in GPC3 positive group, while there is only one (1/8) in GPC3 negative group.

Discussion

In recent years, preliminary investigations of aberrant expression of GPC3, Arg-1 and HepPar-1 in HCC have been reported. Evidences prove that GPC3, Arg-1 and HepPar-1 plays important roles in progression and metastasis of HCC. Such as, GPC3 promotes proliferation and invasion of HCC (22,23). ARG1 might play a key role in progression of HCC via promoting the epithelial-to-mesenchymal transition (EMT) process (24). Suppression of the expression of Hep Par1 in liver cancer cells (SMMC-7721) inhibited cell proliferation (25). Collectively, these reports provide us a glimpse of dynamic involvements of

Table 4 The expression level of GPC3, Arg-1, and HepPar-1 in different tissues

Cancer kind	Total	GPC3				Arg-1				HepPar-1			
		0 (–)	1+	2+	3+	0 (–)	1+	2+	3+	0 (–)	1+	2+	3+
HCC	47	15	6	23	3	11	5	25	6	10	8	14	15
Well-differentiated	19	5	2	10	2	5	3	8	3	4	1	7	7
Moderately differentiated	20	7	2	10	1	3	2	12	3	3	5	6	6
Poorly differentiated	8	3	2	3	0	3	0	5	0	3	2	1	2
ICC	29	27	1	1	0	27	1	1	0	25	1	3	0
Well-differentiated	4	4	0	0	0	4	0	0	0	3	0	1	0
Moderately differentiated	17	16	1‡	0	0	15	1	1‡	0	15	1	1‡	0
Poorly differentiated	8	7	0	1	0	8	0	0	0	7	0	1	0
Paracancerous tissue	76	74	2	0	0	1	3	38	34	2	7	39	28

‡, represented the sample BC99. GPC3, glypican-3; Arg-1, arginase-1; HepPar-1, hepatocyte paraffin antigen 1; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

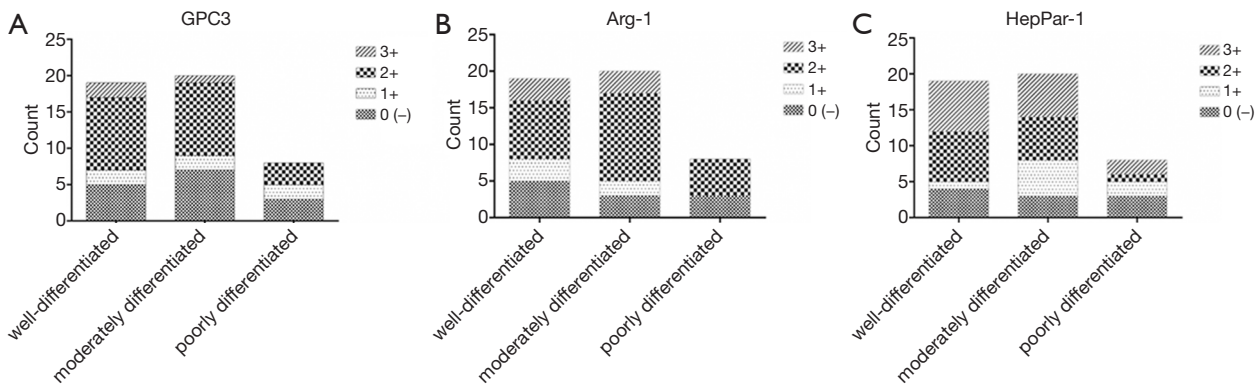

Figure 2 The distribution of positive levels in different differentiated HCC tissues for different biomarkers. (A) The expression level distribution of GPC3 in different differentiated HCC tissues; (B) the expression level distribution of Arg-1 in different differentiated HCC tissues; (C) the expression level distribution of HepPar-1 in different differentiated HCC tissues (*Figure S1*). HCC, hepatocellular carcinoma; GPC3, glypican-3; Arg-1, arginase-1; HepPar-1, hepatocyte paraffin antigen 1.

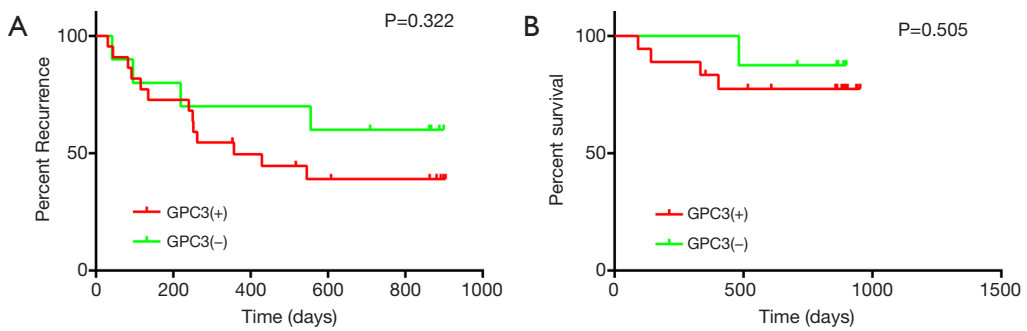

Figure 3 The recurrence and survival analysis for GPC3 expression in HCC. (A) DFS results for recurrence; (B) OS results for survival. GPC3, glypican-3; HCC, hepatocellular carcinoma.

GPC3, Arg-1 and HepPar-1 in HCC and may as potential biomarkers and therapeutic targets. Our follow-up results showed that the recurrence rate in GPC3 positive group seemed little higher than that in GPC3 negative group. Though no statistical significance was found, it may due to the small sample size and higher rate of loss to follow-up.

Distinction of HCC from non-hepatocellular-associated carcinoma is challenging, particularly in cases of poorly differentiated HCC. Various biomarkers, such as Arg-1, HepPar-1, CD34, GP73 and HSP70, have been proposed for distinguishing HCC from other types of liver cancer. However, none of them have been reported to be sufficient (26-28). Arg-1 and HepPar-1, the two most sensitive biomarkers for HCC (with a positive rate of 60–100%), have been turned up with a very poor specificity (29-31). GPC3, a member of the glypican family of heparin sulfate proteoglycans, has emerged as a new promising HCC diagnostic biomarker with positive expression rate of 70–90% and specificity of <90% (32-34). HepPar-1 is recognized as a traditional hepatocyte-specific marker. GPC3 and Arg-1 are relatively new diagnostic biomarkers that are highly expressed in HCC and considered useful in differentiating HCC from metastatic tumors or ICC. An increasing number of studies have reported GPC3 as a new HCC diagnostic biomarker both for early diagnosis or companion diagnosis, due to its advanced specificity and sensitivity. Immunostaining for GPC3, HSP70 and glutamine synthetase and/or gene expression (GPC3, LYVE1 and survivin) is recommended by the EASL-EORTC Clinical Practice Guidelines on the management of HCC for the differentiation between high grade dysplastic nodules and early HCC (35). In our study, the sensitivity and specificity of GPC3 was both superior to those of Arg-1 and HepPar-1. Furthermore, the combination of GPC3, Arg-1 and Par-1 had the best specificity and an improved sensitivity in diagnosing HCC and differentiating HCC from ICC. The aim of this study was to evaluate the diagnostic value of GPC3, Arg-1 and HepPar-1 in HCC, as well as in differentiating HCC from ICC. The results showed that both Arg-1 and HepPar-1 had a high positive expression rate (76.60% and 78.72%, respectively) but poor specificity (2.13% and 0.00%, respectively) for HCC. Even, the positive expression rate of Arg-1 and HepPar-1 in precancerous tissues (98.68% and 97.37%, respectively) was higher than that in HCC. It was consistent with previous reports (36,37). The high specificity of GPC3 and high sensitivity of Arg-1 and HepPar-1 led to the improvement of both sensitivity and specificity in the diagnosis of HCC when GPC3 was combined with Arg-1 or HepPar-1, or Arg-1 and HepPar-1. However, only a small increase of sensitivity was observed in the poorly differentiated HCC subgroup. The small sample size may be the reason for this limitation. It has been previously reported that the positive rate of GPC3 increased along with the decreased differential level in HCC (38,39). In the present study, the positive rate of GPC3 in poorly differentiated HCC was a slightly lower than that in moderate- or well-differentiated HCC. The same distribution trends were observed in Arg-1 and HepPar-1. It appears that all three biomarkers are insufficient for poorly differentiated HCC.

As for distinguishing HCC from ICC, all three biomarkers performed well. The combined panel of markers seems to have little improved effect in distinguishing HCC from ICC compared with single marker. Of note, sample BC99, which positively expressed GPC3, Arg-1 and HepPar-1, was originally classified as ICC and later re-evaluated as HCC. Furthermore, 2 samples that reported negative for Arg-1 and HepPar-1, which originally classified as HCC, were later re-evaluated as non-HCC. This may, or may not be a coincidence. The complete positive results were shown for all the three biomarkers (GPC3, Arg-1 and HepPar-1), and the PPV may indicate for HCC more accurate (PPV, 100%; *Table 4*). The diagnosis of ICC should be made carefully, and re-evaluation may be required. Conversely, negative results for both Arg-1 and HepPar-1 might suggest the possibility of non-HCCs. More consideration should therefore be taken in clinical practice. A limitation of this study was the insufficient statistical power derived from the small sample size. So here we presented the results descriptively without statistically significant. And we would prefer to present these results as phenomena or clues other than as conclusions. Studies with a larger sample size and more types of cancer may provide more information in searching for new biomarkers and new diagnostic methods (40,41).

The highlights of the present study were as follows: (I) the biomarkers examined in this study, particularly GPC3 and Arg-1in, are recently reported biomarkers in the pathological diagnosis of early HCC and differential diagnosis of HCC, cholangiocarcinoma and metastatic carcinoma of the liver (42,43). (II) The combination of GPC3, Arg-1 and HepPar-1 in differentiating HCC from ICC is a relatively new method that has not been sufficiently studied (12,15,19). Herein, the diagnostic and differential diagnostic values of these three markers in single or different combined styles were validated in different classified tissues, according to pathological

results, respectively. The expression rate and level of GPC3, Arg-1 and HepPar-1 in different origin tissues (HCC, ICC, and their adjacent tissues) and different differentiated HCC tissues were all examined. It was found that the expression intensity of HepPar-1 in poorly differentiated HCC was much higher than that of GPC3 and Arg-1. (III) Since GPC3 is a relatively new biomarker, there is no standard monoclonal antibody for it. Even though the commercial monoclonal antibody 1G12 can be purchased and has been included in several studies, it does not mean that there are no better options. Yasuda *et al.* used a commercial ELISA kit with a GPC3 antibody 1G12 and reported that it did not perform well in diagnosing HCC (44). In the present study, the 7D11 monoclonal antibody 7D11 was used (our patent product, ZL201210086009.X, http://cpquery.sipo.gov.cn/) (20); it has been confirmed as an useful regent for GPC3 detecting by IHC (45), chemiluminescent immunoassay (41,46) and time-resolved fluorescence immunoassay (40). Our previous study showed that the 7D11 antibody was equivalent to 1G12 as a regent for GPC3 detection by IHC (45). The patent for the monoclonal antibody 7D11 was transferred to Darui Biotechnology, Guangzhou on Aug 23, 2017, and it is now available for commercialized use by Darui Biotechnology.

In conclusion, the comparison between GPC3 and Arg-1 or HepPar-1 suggested that the combination of GPC3, Arg-1 and Par-1 showed the best specificity and reasonable sensitivity for HCC. GPC3, Arg-1 and HepPar-1 were all useful biomarkers in differentiating HCC from ICC. The combination models can improve the values of any of these markers individually, both in diagnosing HCC and in differentiating HCC from ICC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.11.20). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethical Committee of the First Affiliated Hospital of Zhejiang University (REC number: EC-2015-82). The specimens were obtained with the consent of the patients.

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Supplementary

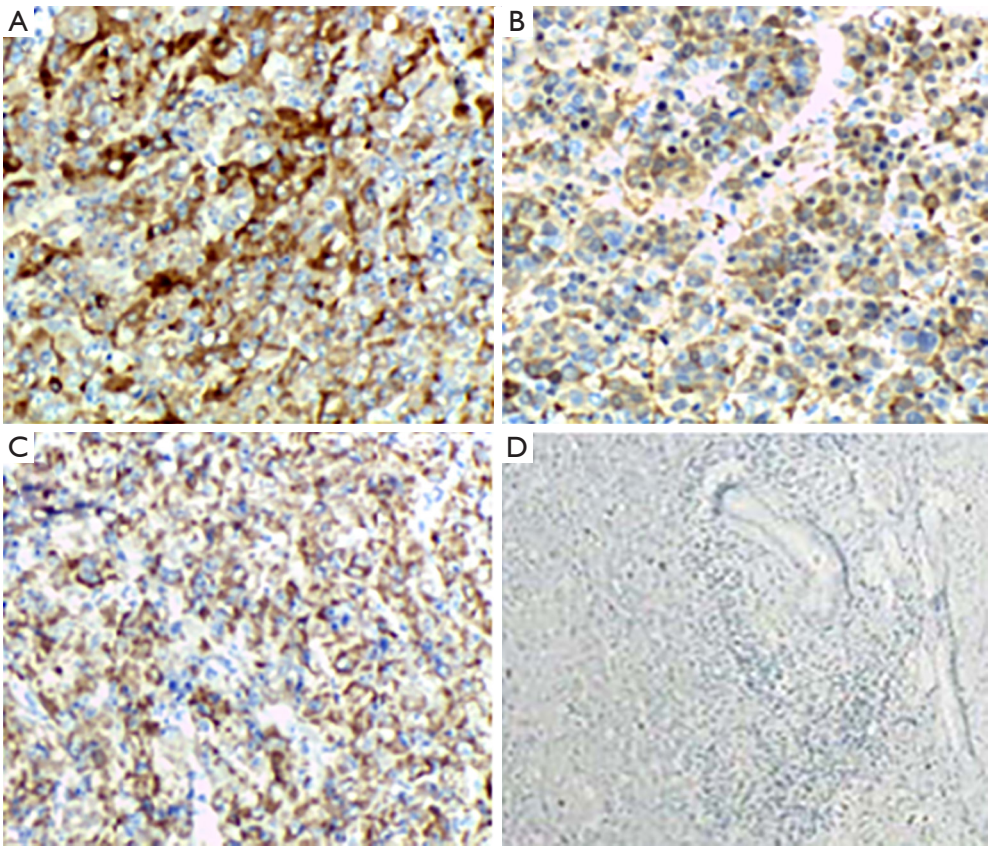

Figure S1 Representative expression images of GPC3, Arg-1, HepPar-1 in HCC by immunohistochemically stained: (A) GPC3 (×200); (B) Arg-1 (×200); (C) HepPar-1 (×200); (D) negative control (PBS, ×80). GPC3, glypican-3; Arg-1, arginase-1; HepPar-1, hepatocyte paraffin antigen 1; HCC, hepatocellular carcinoma.