RESEARCH ARTICLE

CK19 and Glypican 3 Expression Profiling in the Prognostic Indication for Patients with HCC after Surgical Resection

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Abstract

This retrospective study was designed to investigate the correlation between a novel immunosubtyping method for hepatocellular carcinoma (HCC) and biological behavior of tumor cells. A series of 346 patients, who received hepatectomy at two surgical centers from January 2007 to October 2010, were enrolled in this study. The expressions of cytokeratin 19 (CK19), glypican 3 (GPC3), and CD34 were detected by immunohistochemical staining. The clinical stage was assessed using the sixth edition tumor–node–metastasis (TNM) system (UICC/AJCC, 2010). Vascular invasion comprised both microscopic and macroscopic invasion. The tumor size, lymph node involvement, and metastasis were determined by pathological as well as imaging studies. Recurrence was defined as the appearance of new lesions with radiological features typical of HCC, seen by at least two imaging methods. Survival curves for the patients were plotted using the Kaplan–Meier method, and differences between the curves were assessed using the log-rank test. Significant differences in morphology, histological grading, and TNM staging were observed between groups. Based on the immunohistochemical staining, the enrolled cases were divided into CK19+/GPC3+, CK19−/GPC3+ and CK19−/GPC3− three subtypes. CK19+/GPC3+ HCC has the highest risk of multifocality, microvascular invasion, regional lymph node involvement, and distant metastasis, followed by CK19−/GPC3+ HCC, then CK19−/GPC3− HCC. CK19+/GPC3+ HCC has the shortest recurrence time compared to other immunophenotype HCCs. CK19 and GPC3 expression profiling is an independent prognostic indicator in patients with HCC, and a larger sample size is needed to further investigate the effect of this immunosubtyping model in stratifying the outcome of HCC patients.
Introduction

Hepatocellular carcinoma (HCC) is the third most deadly malignant tumor in the world. In China, the incidence of this malignancy is approximately 55% of the global incidence. Liver resection is the first-line therapy in patients with solitary tumor and well-preserved liver function. However, high recurrence rate greatly impact the curative effect after hepatectomy. Many independent risk factors, including the tumor size, nodule number, histological grading, and vascular invasion, were identified to be closely associated with the recurrence and survival of HCC patients [1,2]. In the molecule level, the relationship between the expression of some proteins, such as CD133, OV6, CD44, CD47, CK19, and EpCAM, and poorer outcomes of HCC was also established [3–6]. Of note, almost all of these molecules were biomarkers for hepatic stem cell (HSC) or hepatic progenitor cell (HPC).

Traditionally, HCC has long been believed to be transformed from the mature hepatocytes by dedifferentiation process. With the understanding of the hierarchical makeup of parenchymal cells in the adult normal liver or chronic liver diseases, it was recognized that HCC consisted of a heterogeneous group of subtypes, which may have transformed from HPC, as well as the progenies of HPC [7,8]. The relationship between the expression of HSC or HPC biomarkers and poor prognosis of HCC patients prompts us to speculate whether the aggressiveness of tumor cell is associated with the differentiation status of tumor cell before malignant transformation.

To prove the hypothesis, a panel of biomarkers for the distinction of differentiation status of tumor cell is crucial. HPC overexpresses CK19 and possesses a strong bidirectional differentiation potential toward the biliary and hepatic lineages. When committed to hepatic lineage, HPC decreases the expression of CK19 at the very early stage of differentiation [9–10]. However, cholangiocyte can constantly express CK19. Therefore, CK19 is currently well-accepted as a biomarker for HCC with HPC origin, besides the cholangiocyte carcinoma. According to the published literatures, CK19+ HCC accounts for nearly 20% of all HCCs [3,4].

Fetal protein glypican 3 (GPC3) is another biomarker for HPC, as well as immature hepatocytes. Only at the terminal differentiation period of HPC toward mature hepatocyte, the expression of GPC3 was absent [11,12]. In addition, GPC3 is never expressed in cholangiocyte, cholangiocarcinoma, and low expressed in well-differentiated HCC [13,14]. It was reported that GPC3+ HCC accounts for nearly 72%–81% of all HCCs [15]. According to the specific expression phase and spectrum of CK19 and GPC3 in the differentiation process of HPC toward mature hepatocyte, HCC was subclassified as CK19+/GPC3+, CK19−/GPC3+, and CK19−/GPC3−-phenotypes, which roughly corresponded to HCC subtype transform from the HPC, immature hepatocyte, and terminal differentiated hepatocyte, respectively.

Although previous reports have shown the significance of the expression of CK19 [16,17] or GPC3 [18,19] in the prognosis evaluation of HCC patients, in view of the specific expression spectrum of the proteins, combined detection of the two markers can better indicate the differentiation status of tumor cells than utilization of them individually. In the present study, the correlation between the immunosubtyping method of HCC based on the CK19 and GPC3 expression profiling and biological behavior of tumor cells were investigated. Furthermore, the value of this molecular subtyping model in the risk stratification of HCC patients was tested.

Patients and Methods

The study was approved by the Ethical Committee of Beijing You-An Hospital, Capital Medical University. All methods and procedures associated with this study were accorded ethically with the principles of the Declaration of Helsinki and local laws. All patients provided written
informed consent before enrollment. All authors had access to the study data and reviewed and approved the final manuscript.

Study Design
This was a retrospective study of patients with HCC admitted for hepatectomy. From January 2007 and October 2010, a total of 346 patients, who received a first-time diagnosis of HCC and eventually received pathologic confirmation of the diagnosis, at You-An Hospital, Capital Medical University, and the 304 Hospital, PLA, Beijing, P.R. China, were evaluated.

The entry criteria included the following: (1) the entire tumor (including the main tumor, satellites, and multicenter tumors; at least from the edge of the tumor margins > 1cm) was resectable; (2) liver function was classified as grade A of the Child-Pugh classification; (3) no distant metastases were found (three suspected metastasis cases were identified to be metastases after surgical resection during the follow up); and (4) the remnant volume of the liver was considered adequate and no contraindication to laparotomy was found. The exclusion criteria were as follows: (1) patients with other malignancies; (2) patients with liver function Child–Pugh B and C; (3) patients with less than 1 month of life expectancy or follow-up; (4) patients with uncontrolled severe diabetes and acute infection; and (5) patients having no history of chemotherapy or radiotherapy before surgery. Clinical data were extracted from the clinical charts. For comparability among different subtypes of HCC, the clinical stage was assessed using the sixth edition tumor–node–metastasis (TNM) system (UICC/AJCC, 2010) [20]. Vascular invasion comprised both microscopic and macroscopic invasion. The tumor size, lymph node involvement, and metastasis were determined by pathological as well as imaging studies.

Follow-Up
All patients were followed for 1 month after hepatectomy, then every 3 months during the first year after surgery, and every 6 months thereafter. During follow-up visits, patients were subjected to physical examination, liver function tests, abdominal ultrasonography, and computed tomography or magnetic resonance imaging of the liver. Recurrence was defined as the appearance of new lesions with radiological features typical of HCC, seen by at least two imaging methods. The patients were followed up for at least 36 months. Recurrence-free survival (RFS) was calculated as the time from the date of surgery until the date of tumor recurrence and was censored at the time of last following-up or death if at that time there was no evidence of tumor recurrence.

Hematoxylin–Eosin (HE) and Immunohistochemical Stains
Routine HE staining and immunohistochemistry staining were performed as described in a previous study [21]. Mouse anti-human CK19 monoclonal antibody (Clone BA17; dilution, 1:100) and Mouse anti-human GPC3 monoclonal antibody (Clone 1G12; dilution, 1:200) were purchased from Zeta Corporation (Sierra Madre, CA, USA). Mouse anti-human monoclonal antibody CD34 (Clone QBEnd/10; dilution, 1:100) was purchased from Zymed Laboratories (San Francisco, CA, USA). Steamer for 20 minutes in citrate target retrieval buffer (pH 6.0). The results of immunohistochemical staining were considered positive if greater than 10% of the tumor cells showed cytoplasmic staining for CK19, GPC3, or CD34. Probably due to the spontaneous differentiation, in some of the CK19 or GPC3 positive HCC, patchy positive for CK19 or GPC3 staining in varying degrees could be observed. In these cases, when the tumor cells co-expression CK19 and GPC3 (more than 5%), they were classified into the CK19+/GPC3+ group [3,19]. Histologically, all of the primary carcinoma of the liver with CK19+/GPC3− phenotype in our center meets the diagnostic criteria of the intrahepatic cholangiocarcinoma, which were excluded from current analyses. Therefore, all enrolled cases were divided into three groups:
CK19+/GPC3+ group, included cases where tumor cells coexpress CK19 and GPC3; CK19−/GPC3+ group, included cases where tumor cells express GPC3 singly; and CK19−/GPC3− group, included cases with negative expression of both CK19 and GPC3. The evidence of cytoplasmic staining of adjacent interlobular duct epithelia served as internal positive control for CK19; yolk sac tumor tissue was used as a positive control sample for GPC3. Negative controls were carried out by substitution of the primary antibodies with nonimmunized serum, which resulted in no signal detection. The expression of these markers was assessed independently and blindly by two consultant histopathologists (JF and FL). All slides were reviewed to confirm the diagnosis according to the guidelines of the World Health Organization (WHO) criteria, 2010 [20]. CD34 staining was helpful in the recognition of a trabecular pattern.

Statistical Analysis
All analyses were performed using the SPSS 13.0 software (SPSS Inc., IL, USA). The data were expressed as mean ± standard deviation. The chi-square test and Student t-test were applied to compare the distribution of categorical and continuous variables between the groups, respectively. Survival curves for the patients were plotted using the Kaplan–Meier method and differences between the curves were assessed using the log-rank test. The Cochran–Armitage trend test was used to study the underlying trend. Linear regression with collinearity diagnostics was performed for data correlated with RFS. A variance inflation factor (VIF) higher than 10 was indicative of high collinearity. Univariable and multivariable Cox proportional hazards regression analysis was used to assess factors associated with recurrence of HCC. \( P < 0.05 \) was considered statistically significant.

Results
During the study period, 422 HCC patients were treated at the two hospitals. Of these, 63 (14.93%) were excluded because they had received local ablation therapy, ethanol injection, or transarterial chemoembolization before the hepatectomy. And, another 13 (3.08%) were excluded because they did not participate in the follow-up. The remaining 346 (81.99%) patients satisfied the inclusion criteria and were included in the present study. Of these, 121 (34.97%), 47 (13.58%), and 20 (5.78%) experienced recurrence within 1, 2, and 3 years after hepatectomy, respectively. The other 19 (5.49%) experienced recurrence beyond 3 years after hepatectomy. The IHC staining showed 69 CK19+/GPC3+ cases, 224 CK19−/GPC3+ cases, and 53 CK19−/GPC3− cases accounting for 19.94%, 64.74%, and 15.32% of all enrolled patients, respectively.

Baseline Characteristics
As shown in Table S1, the average onset age of CK19+/GPC3+ HCC was earlier than that of the other subtypes, but difference between CK19−/GPC3+ and CK19−/GPC3− HCC was not significant. All HCCs were more common in males than females. A significant difference in gender distribution was not found between any two HCC subtypes. Significant differences in underlying cirrhosis between any two HCC subtypes were not found.

Relationship between Immunosubtype and Histological Features of HCC

Correlation between immunosubtype and morphological classification or histological grading of HCC. Morphologically, acinar/thin trabecular HCC (57/346), thick trabecular HCC (243/346), and compact variants (12/346) were predominant in the present series.
Pseudoglandular variant usually present as a component of thin trabecular HCC, therefore they were combined into the same group. Thirty-four scirrhous HCCs (SHCCs), a subtype of HCC characterized by diffused fibrosis along the sinusoid-like blood spaces, were found in the present series [22]. Majority of SHCCs (31/34) showed CK19+/GPC3+ expression. Thirty-three CK19+/GPC3+ cases showed abundant fibrous stroma. According to the parenchymal cell to fibrous stroma ratio, they do not fully meet the diagnostic criteria of SHCC. However, fibrous stroma among the tumor nests was very rich compared to that in CK19−/GPC3+ and CK19−/GPC3− groups.

Thick trabecular and scirrhous variants accounted for 47.83% (33/69) and 44.93% (31/69) of all CK19+/GPC3+ cases, respectively. Compared to that of the acinar/thin trabecular and compact variants, the difference in the distribution of the thick trabecular or scirrhous variants in CK19+/GPC3+ HCC was significant (P<0.01; P<0.01). Tumors with thick trabecular pattern accounted for 91.96% (206/224) of all CK19−/GPC3− HCC, which was significantly higher than that of other histological variants in the CK19−/GPC3+ group (P<0.01). Acinar/thin trabecular variants accounted for 83.02% (44/57) of all CK19−/GPC3− HCC cases, which was significantly higher than that of the other histological variants in the CK19−/GPC3− group (P<0.01). These results showed close correlation between the immunosubtype and morphological classification of HCC. CK19+/GPC3+ HCCs were more prone to present with thick trabecular pattern accompanied by fibrous stroma. CK19−/GPC3− HCCs more likely appeared in the acinar/thin trabecular pattern, which mimicked the normal hepatic plates. And CK19−/GPC3+ HCCs more likely presented in the thick trabecular pattern (Table 1; Fig 1).

Well-differentiated HCC usually presented in the thin trabecular pattern (1–3 hepatocytes thick) and minimal nuclear atypia. CK19−/GPC3− and CK19−/GPC3+ cases accounted for about 59.09% and 40.91% of all well-differentiated HCC patients, respectively. No CK19+/GPC3+ cases presented in the well-differentiated HCC group. Compared to that in the moderately or poorly differentiated HCC, lower percentage of CK19−/GPC3+ cases in well-differentiated HCC were found (P<0.01; P<0.01). Compared to that in the CK19−/GPC3+ or CK19+/GPC3+ groups, the percentage of poorly differentiated cases were significantly lower in the CK19+/GPC3− group (P<0.01; P<0.01). The tendencies were observed in the distribution of the well- or poorly differentiated HCC groups along with the CK19+/GPC3+, CK19−/GPC3+, CK19−/GPC3− phenotype (P_trend<0.01; P_trend<0.01). However, the difference in the distribution of moderately differentiated HCC between any two immunophenotype HCC was not significant (P>0.05; P>0.05; P>0.05) (Table 2).

**Multicollinearity analysis.** Since the regression coefficients may be compromised by collinearity, the VIF was checked as an indicator for collinearity. Linear regression with

| Histological variation | CK19+/GPC3+ | CK19−/GPC3+ | CK19−/GPC3− |
|------------------------|------------|-------------|-------------|
| Acinar/thin trabecular | 4 (5.80)   | 9 (4.02)    | 44 (83.02)  |
| Thick trabecular      | 33 (47.83) | 206 (91.96) | 4 (7.55)    |
| Compact                | 1 (1.45)   | 8 (3.57)    | 3 (5.66)    |
| Scirrhous              | 31 (44.93) | 1 (0.41)    | 2 (3.77)    |
| n                      | 69         | 224         | 53          |
| X²                     | 523.31     | 159.46      | 206.45      |
| P                      | P<0.01     | P<0.01      | P<0.01      |

Data are given as number (percentage). HCC, hepatocellular carcinoma; CK19, cytokeratin 19; GPC3, glypican 3

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**Table 1. The relationship between the histological variation and immune-subtypes of HCC.**

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collinearity diagnostics showed collinearity didn’t exist within the immunophenotype, histological classification, and grading degree of HCC (VIF = 1.097). Therefore, these correlated factors can be regarded as the major factors.

![Fig 1. CK19 and GPC3 expression in different histological variation of HCC.](image)

**Table 2. The correlation between immune-phenotype and histological grading of HCC.**

| Immuno-phenotype   | Well   | Moderately | Poorly  | n   |
|--------------------|--------|------------|---------|-----|
| CK19+/GPC3+        | 0 (0.00) | 26 (37.68) | 43 (62.32) | 69  |
| CK19-/GPC3+        | 9 (4.02) | 102 (45.54) | 113 (50.45) | 224 |
| CK19-/GPC3-        | 13 (24.53) | 25 (47.17) | 15 (28.30) | 53  |
| CK19+/GPC3+ vs. CK19-/GPC3+ | $X^2 = 1.67 \ P > 0.05$ | $X^2 = 1.32 \ P > 0.05$ | $X^2 = 2.99 \ P > 0.05$ |  |
| CK19+/GPC3+ vs. CK19-/GPC3- | $X^2 = 18.94 \ P < 0.01$ | $X^2 = 1.11 \ P > 0.05$ | $X^2 = 13.91 \ P < 0.01$ |  |
| CK19-/GPC3+ vs. CK19-/GPC3- | $X^2 = 21.93 \ P < 0.01$ | $X^2 = 0.05 \ P > 0.05$ | $X^2 = 8.46 \ P < 0.01$ |  |

Data are given as number (percentage). HCC, hepatocellular carcinoma; CK19, cytokeratin 19; GPC3, glypican 3

![DOI:10.1371/journal.pone.0151501.g001](image)

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Relationship between the immunosubtype and TNM or clinical staging of HCC

As shown in Table 3, in stage I HCC, patients with CK19−/GPC3+ expression took up the highest percentage (49.06%), then CK19+/GPC3+ (41.07%) and CK19+/GPC3− (17.39%) (0.01 < P < 0.05). However, in stage IV HCC, the result was just the opposite (P < 0.01). The difference in the distribution of the three immunophenotype HCC in stage II or III group was not statistically significant.

The tendencies in the increase of the percentage of CK19+/GPC3+ cases and decrease of the percentage of CK19+/GPC3− and CK19−/GPC3+ cases along with the clinical staging from I to IV were also observed (P_trend < 0.01; P_trend < 0.05; P_trend < 0.05).

As shown in Table 4, the differences in multifocality, microvascular invasion, regional lymph node involvement, and distant metastasis between CK19+/GPC3+ and CK19−/GPC3+ or CK19−/GPC3− groups were significant. In addition, the difference in microvascular invasion between CK19−/GPC3+ and CK19−/GPC3− groups was also significant. The Cochran–Armitage trend test showed that the rates of multifocality, microvascular invasion, regional lymph node involvement, and distant metastasis increased significantly along with the alteration of phenotype from CK19−/GPC3− to CK19−/GPC3+, then CK19+/GPC3+ (P_trend < 0.01; P_trend < 0.01; P_trend < 0.01; P_trend < 0.01). These results showed that the malignant behavior of HCCs was closely associated with their immunophenotype. CK19+/GPC3+ HCC was the most aggressive subtype, followed by the CK19−/GPC3+ HCC. CK19−/GPC3− HCC was the least aggressive subtype.

| Immuno-phenotype | I     | II    | III   | IV    | n  | X^2  | P     |
|------------------|-------|-------|-------|-------|----|------|-------|
| CK19+/GPC3+      | 12(17.39) | 26(37.66) | 23(33.33) | 8(11.59) | 69 | 17.50 | P<0.01|
| CK19−/GPC3+      | 92(41.07) | 76(33.93) | 53(23.66) | 3(1.34)  | 224| 2.39  | P>0.05|
| CK19−/GPC3−      | 26(49.06) | 17(32.08) | 10(18.87) | 0(0.00)  | 53 | 3.99  | P>0.05|
| n                | 130   | 119   | 86    | 11     | 346|

Data are given as number (percentage). HCC, hepatocellular carcinoma; CK19, cytokeratin 19; GPC3, glypican 3

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| Phenotype | Multifocality | Microvascular Invasion | Involvement of major branch of vein | Liver capsule invasion | Regional lymph node involvement | Distant metastasis | n    |
|-----------|---------------|------------------------|------------------------------------|------------------------|---------------------------------|-------------------|------|
| CK19+/GPC3+ vs. CK19−/GPC3+ | X^2 = 10.03 | X^2 = 6.27 | X^2 = 2.16 P > 0.05 | X^2 = 0.60 | X^2 = 4.27 | X^2 = 12.65 | 69   |
|              | P < 0.01     | P < 0.01              | P > 0.05                          | P > 0.05               | 0.01 < P < 0.05         | P < 0.01         |      |
| CK19+/GPC3+ vs. CK19−/GPC3− | X^2 = 12.27 | X^2 = 17.24 P < 0.01 | X^2 = 0.32 P > 0.05 | X^2 = 0.57 | X^2 = 3.98 | X^2 = 4.82 | 224  |
|              | P < 0.01     |                      |                                  | P < 0.01               | 0.01 < P < 0.05         |                  |      |
| CK19−/GPC3+ vs. CK19−/GPC3− | X^2 = 2.37 | X^2 = 7.48 P < 0.01 | X^2 = 0.00 P > 0.05 | X^2 = 0.00 | X^2 = 0.67 P < 0.01 | X^2 = 0.01 | 53   |
|              | P > 0.05     |                      |                                  | P > 0.05               | P < 0.05                |                  |      |

Data are given as number (percentage). TNM, the TNM (Tumor, Node, Metastasis) staging system; CK19:cytokeratin 19; GPC3:glypcan3

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Univariate Analysis with Respect to Tumor Recurrence

The total recurrence rate of patients was 59.8%. Median time to recurrence was 23 months. RFS was compared for 11 possible prognostic factors, including age, gender, presence of cirrhosis, multiplicity of tumors, greatest tumor dimension, immunophenotype, microvascular invasion, involvement of major branch of vein, perforation of visceral peritoneum, regional lymph node involvement, distant metastasis, histological grading, and the American Joint Committee on Cancer (AJCC) clinical staging. Using the Kaplan–Meier method, univariate analysis showed that multiplicity, microvascular invasion, macrovascular invasion, histological grading, immunophenotype, histological grading, AJCC clinical staging, and regional lymph node involvement were significantly associated with the RFS of patients (Table 5).

Median time to recurrence of CK19+/GPC3+, CK19−/GPC3+, and CK19−/GPC3− groups was 10, 26, and 43 months, respectively. The corresponding 1-, 2-, and 3-year survivals were 44.49%, 24.24%, and 16.52%; 68.82%, 52.10%, and 40.46%; and 69.13%, 57.94%, and 49.35%, respectively. The log-rank test showed a significant difference among the survival curves of the three subtypes ($P < 0.001$). The cumulative RFS rate was significantly higher in CK19−/GPC3+ and CK19−/GPC3− groups than in the CK19+/GPC3+ group ($P < 0.01$; $P < 0.01$). However, the cumulative RFS rate was similar between CK19−/GPC3+ and CK19−/GPC3− groups ($P > 0.05$) (Fig 2).

Multivariable Cox Proportional Hazards Regression Analysis of Risk Factors Associated with Recurrence

The seven factors that were significant in the univariate analysis were entered in the multivariate analysis as shown in Table 6. Only immunophenotype [hazard ratio (HR) = 0.709, 95% confidence interval (CI) = 0.545–0.921, $P = 0.01$], histological grading (HR = 0.740, 95% CI = 0.569–0.962, $P < 0.05$), multiplicity of tumors (HR = 1.572, 95%CI = 1.137–2.174, $P < 0.01$), and microvascular invasion (HR = 1.509, 95%CI = 1.074–2.120, $P < 0.05$) were shown to be independent predictors of RFS.

Discussion

In the present study, it was found that from CK19+/GPC3+ HCC to CK19−/GPC3+ HCC, and then CK19−/GPC3− HCC, tumor cell in intrahepatic metastasis, microvascular invasion, regional lymph node involvement, and distant metastasis was decreased. In addition, the multivariate analysis showed that this immune subclassification method of HCC was an independent predictor of RFS. These evidences supported that the cellular differentiation status was closely linked to the aggressive potential of the tumor. Of note, the term “differentiation” in this study was restored to its meaning in developmental biology, which is distinct from the same term used commonly in the oncology research field to describe the histological grading degrees based on the morphological observation. Although closely correlated, collinearity diagnosis showed no collinearity problem between the two factors, which indicated that both cellular differentiation status and histological grading were independent predictors of RFS.

Edmondson-Steiner grading system is the most commonly used method for the histological grading of HCC. In this system, cellular and architectural atypia is the crucial criteria. Since tumorigenesis and progression were principally a consequence of accumulated mutations, a striking atypia of tumor means more accumulated mutations a tumor acquired [23–25], which can be the reason for the correlation between histological grading and HCC recurrence. However, this genetic alteration mechanism cannot fully explain why the aggressive behavior of tumor cells is closely associated with the cellular status of differentiation in HCC.
Table 5. Univariate analysis with respect to tumor recurrent.

| Characteristic | n   | 6 month | 12 month | 24 month | 36 month | P value |
|----------------|-----|---------|----------|----------|----------|---------|
| Gender         |     |         |          |          |          |         |
| Male           | 283 | 76.210  | 63.269   | 45.593   | 34.178   | 0.155   |
| Female         | 63  | 87.004  | 70.124   | 53.601   | 50.218   |         |
| Age            |     |         |          |          |          | 0.0975  |
| <50            | 125 | 77.033  | 57.925   | 39.091   | 27.567   |         |
| >50            | 211 | 82.203  | 68.271   | 51.443   | 42.079   |         |
| Cirrhosis      |     |         |          |          |          | 0.2238  |
| Yes            | 273 | 76.798  | 62.906   | 44.399   | 37.043   |         |
| No             | 73  | 83.349  | 70.635   | 47.341   | 36.946   |         |
| Size of main nodule (cm) |     |         |          |          |          |         |
| <3             | 171 | 82.981  | 66.315   | 48.815   | 36.867   | 0.1323  |
| 3<5<5          | 72  | 77.512  | 70.466   | 53.607   | 41.755   |         |
| >5             | 103 | 70.515  | 57.260   | 38.914   | 33.712   |         |
| Nodule number  |     |         |          |          |          | <0.0001 |
| 1              | 251 | 82.411  | 70.739   | 52.350   | 41.609   |         |
| 1<n<3          | 95  | 66.714  | 47.549   | 32.107   | 23.711   |         |
| Microvascular invasion |     |         |          |          |          | <0.0001 |
| Yes            | 158 | 65.373  | 50.707   | 32.472   | 25.215   |         |
| No             | 188 | 88.794  | 75.927   | 58.369   | 46.192   |         |
| Macrovascular invasion |     |         |          |          |          | <0.0014 |
| Yes            | 20  | 58.438  | 31.875   | 15.938   | 15.938   |         |
| No             | 326 | 79.323  | 66.449   | 48.853   | 38.098   |         |
| Histological grading |     |         |          |          |          | <0.0002 |
| Poorly         | 171 | 68.034  | 55.260   | 35.175   | 28.341   |         |
| Moderately     | 153 | 87.532  | 73.589   | 55.124   | 42.824   |         |
| Well           | 22  | 90.911  | 72.727   | 72.727   | 57.132   |         |
| Immuno-phenotype |   |         |          |          |          | <0.0001 |
| CK19+/GPC3+    | 69  | 57.155  | 44.494   | 24.235   | 16.524   |         |
| CK19-/GPC3+    | 224 | 82.927  | 68.818   | 52.103   | 40.460   |         |
| CK19-/GPC3-    | 53  | 84.615  | 69.127   | 57.937   | 49.354   |         |
| Clinical staging (6th AJCC) |     |         |          |          |          | <0.0001 |
| I              | 130 | 90.000  | 77.685   | 66.615   | 52.891   |         |
| II             | 119 | 78.047  | 62.359   | 34.100   | 22.627   |         |
| III            | 86  | 60.986  | 47.569   | 31.443   | 28.242   |         |
| IV             | 11  | 71.616  | 61.364   | 51.124   | 40.921   |         |
| Perforation of visceral peritoneum |     |         |          |          |          | 0.7130  |
| Yes            | 26  | 80.841  | 65.385   | 45.313   | 45.313   |         |
| No             | 320 | 77.939  | 64.450   | 47.067   | 36.437   |         |
| Regional lymph node involvement |     |         |          |          |          | 0.0140  |
| Yes            | 14  | 41.734  | 33.317   | 33.317   | 25.013   |         |
| No             | 332 | 79.687  | 65.820   | 50.619   | 37.355   |         |
| Distant metastasis |     |         |          |          |          | 0.4437  |
| Yes            | 14  | 77.938  | 62.338   | 38.961   | 38.961   |         |
| No             | 332 | 78.177  | 64.617   | 47.439   | 36.638   |         |

CK19: cytokeratin 19; GPC3: glypican3; AJCC: American Joint Committee on Cancer

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In normal development, organogenesis, and tissue repair, the whole human genes are well-programmed, subtly modulated, and dynamically expressed, which is beneficial to the development of body and maintenance of organ function. Compared to the terminal differentiated or immature cells, the genetic and epigenetic expression profile in stem or undifferentiated cell is greatly different, which determines the alteration of biological properties and functions, such as cellular adhesion and migration capacities from stem cells to terminal differentiated cells [26,27]. Under carcinogenic stimuli, quite a lot of genetic or epigenetic processes in tumor initiating cells can be altered; however, there still remain an overwhelming majority of normal genetic and epigenetic processes that are not involved and continue into the transformed cells [28–30]. Although these normal events may not be contributors for the onset of malignant transformation, yet could be preserved by the transformed cells and present in an aggressive profile, such as invasion and metastasis. This might be the reason why HCCs with primitive immunophenotype of differentiation are more aggressive.

Table 6. Multivariable Cox proportional hazards regression analysis.

| Characteristic                        | HR     | 95.0% CI      | P value |
|---------------------------------------|--------|---------------|---------|
| Immuno-phenotype                      | 0.709  | 0.545–0.921   | 0.010   |
| Histological grading                  | 0.740  | 0.569–0.962   | 0.025   |
| Nodule number                         | 1.572  | 1.137–2.174   | 0.006   |
| Microvascular invasion                | 1.509  | 1.074–2.120   | 0.018   |
| Macrovascular invasion                | 1.352  | 0.790–2.314   | 0.271   |
| Clinical staging                      | 1.000  | 0.813–1.230   | 0.999   |
| Regional lymph node involvement       | 1.571  | 0.809–3.050   | 0.182   |

HR: hazard ratio; CI: Confidence Intervals
The current work showed that the multiplicity of tumors and microvascular invasion were the independent predictors of RFS for patients with HCCs, which was consistent with several other studies [31]. Multifocal HCC can be of multicentric origin or intrahepatic metastases arising from a primary HCC. Clonal analysis from several independent studies showed that 64%–74.2% of multiple nodules in HCC was intrahepatic metastasis [31–33], which implied, to some degree, the presence of multiplicity under the same background just reflects a stronger invasive capacity of tumor cells. In addition, microvascular invasion can be regarded as essentially intrahepatic metastases, the presence of which also indicates a stronger invasive capacity of tumor cells. Therefore, multiplicity of intrahepatic metastasis, microvascular invasion, regional lymph node involvement, and distant metastasis were just the consequences of aggressive biology of tumor cells. Since the cellular biological property provides intrinsic impetus for the aggressive behavior of tumor cells and information closely linked to the tumor biology provides the most crucial evidence for the assessment of malignancy risk of tumors, compared to the cellular differentiation status and histological grading, the value of multiplicity of intrahepatic metastasis and microvascular invasion in clinical assessment and prognostic evaluation for tumors at early stages is limited.

Currently, the use of some omic techniques has empowered the identification of prognostic subclasses in a wide variety of tumors. In these studies, data retrieved from the omic approaches were all based on the preclassification of patients according to the different clinical outcomes or histological grading degrees of tumors [34]. In the liver, several classification and prognostic predicting systems for HCC were put forward [35–37]. However, they are scarcely used in oncology practice, indicating that these molecular prognostic or predictive tests for HCC have not yet achieved their full potential.

This study has some limitations. The main limitation of this study was the small sample size, particularly the small number of CK19−/GPC3− patients, which resulted in very low power to detect differences in the cumulative RFS rate between the CK19−/GPC3− and CK19−/GPC3+ group patients. In addition, this was a retrospective study conducted at only two centers. A standardized multicenter collaborative study is still needed to highlight the importance of this immuneclassification model in the risk stratification of HCC patients and generate high-level medical evidence so that a uniform set of criteria can be established and recognized.

Conclusions

With the combined detection of CK19 and GPC3, the significance of differentiation status of tumor cells was first revealed in the assessment of aggressive biological behavior of tumor cell. CK19+/GPC3+ HCC has the highest risk of intrahepatic metastasis, microvascular invasion, regional lymph node involvement, and distant metastasis, followed by patients with the CK19−/GPC3+, and then CK19−/GPC3− phenotype. In the current series, although a significant difference in the cumulative RFS rate between CK19+/GPC3+ and CK19−/GPC3+ or CK19−/GPC3− HCC was observed, similar phenomenon between CK19−/GPC3+ and CK19−/GPC3− HCC was not found, which implied that a larger sample size is needed to further investigate the effect of this molecular subtyping method in the risk stratification of HCC patients.

Supporting Information

S1 Checklist. Clinical Studies Checklist.

(Supplementary Material)

S1 Table. Baseline clinical characteristics of all HCC patients included in the study.

(Supplementary Material)
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Author Contributions

Conceived and designed the experiments: JF FL CC. Performed the experiments: SZ LS. Analyzed the data: JF CC FL. Contributed reagents/materials/analysis tools: RZ LY FC GZ FC HX. Wrote the paper: JF RZ.

References

1. Ang SF, Ng ES, Li H, Ong YH, Choo SP, Ngeow J, et al. The Singapore Liver Cancer Recurrence (SLICER) Score for relapse prediction in patients with surgically resected hepatocellular carcinoma. PLoS One. 2015; 10: e0118658. doi: 10.1371/journal.pone.0118658 PMID: 25830231

2. Ikeda K, Saitoh S, Tsubota A, Arase Y, Chayama K, Kumada H, et al. Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma. Cancer. 1993; 71: 19–25. PMID: 8380116

3. Kim H, Choi GH, Na DC, Ahn EY, Kim GI, Lee JE, et al. Human hepatocellular carcinomas with “Stemness”-related marker expression: keratin 19 expression and a poor prognosis. Hepatology. 2011; 54: 1707–1717. doi: 10.1002/hep.24559 PMID: 22045674

4. Lo RC, Ng IO. Hepatic progenitor cells: their role and functional significance in the new classification of primary liver cancers. Liver Cancer. 2013; 2: 84–92. doi: 10.1159/000343844 PMID: 24159600

5. Yang XR, Xu Y, Yu B, Zhou J, Qiu SJ, Shi GM, et al. High expression levels of putative hepatic stem/progenitor cell biomarkers related to tumour angiogenesis and poor prognosis of hepatocellular carcinoma. Gut. 2010; 59: 953–962. doi: 10.1136/gut.2008.176271 PMID: 20442200

6. Yeh CT, Kuo CJ, Lai MW, Chen TC, Lin CY, Yeh TS, et al. CD133-positive hepatocellular carcinoma in an area endemic for hepatitis B virus infection. BMC Cancer. 2009; 9: 324. doi: 10.1186/1471-2407-9-324 PMID: 19744348

7. Wu PC, Fang JW, Lau VK, Lai CL, Lo CK, Lau JY. Classification of hepatocellular carcinoma according to hepatocellular and biliary differentiation markers. Clinical and biological implications. Am J Pathol. 1996; 149: 1167–1175. PMID: 8663666

8. Dom L, Menezes LF, Mikuz G, Otto HF, Onuchic LF, Sergi C. Immunohistochemical detection of polyclonal and co-localization with liver progenitor cell markers during normal and abnormal development of the intrahepatic biliary system and in adult hepatobiliary carcinomas. J Cell Mol Med. 2009; 13: 1279–1290. doi: 10.1111/j.1582-4934.2008.00519.x PMID: 19292732

9. Schmelzer E, Wauthier E, Reid LM. The phenotypes of pluripotent human hepatic progenitors. Stem Cells. 2006; 24: 1852–1858. doi: 10.1634/stemcells.2006-0036 PMID: 16627685

10. Turner R, Lozoya O, Wang Y, Cardinale V, Gaudio E, Alpini G, et al. Human hepatic stem cell and maturation liver lineage biology. Hepatology. 2011; 53: 1035–1045. doi: 10.1002/hep.24157 PMID: 21374667

11. Grozdanov PN, Yovchev MI, Dabeva MD. The oncofetal protein glypican-3 is a novel marker of hepatocellular carcinoma. Lab Invest. 2006; 86: 1272–1284. doi: 10.1038/labinvest.3700479 PMID: 17117158

12. Roskams T, Katoonizadeh A, Komuta M. Hepatic progenitor cells: an update. Clin Liver Dis. 2010; 14: 705–718. doi: 10.1016/j.clid.2010.08.003 PMID: 21055691

13. Li J, Gao JZ, Du JL, Wei LX. Prognostic and clinicopathological significance of glypican-3 overexpression in hepatocellular carcinoma: a meta-analysis. World J Gastroenterol. 2014; 20:6336–6344. doi: 10.3748/wjg.v20.i20.6336 PMID: 24876758

14. Ho M, Kim H. Glypican-3: a new target for cancer immunotherapy. Eur J Cancer. 2011; 47: 333–338. doi: 10.1016/j.ejca.2010.08.024 PMID: 21127733

15. Sawada Y, Yoshikawa T, Shimomura M, Iwama T, Endo I, Nakatsu T. Programmed death-1 blockade enhances the antitumor effects of peptide vaccine-induced peptide-specific cytotoxic T lymphocytes. Int J. Oncol. 2015; 46:28–36. doi: 10.3892/ijo.2014.2737 PMID: 25354479

16. Govaere O, Komuta M, Berkers J, Spee B, Janssen C, de Luca F, et al. Keratin 19: a key role player in the invasion of human hepatocellular carcinomas. Gut. 2014; 63: 674–685. doi: 10.1136/gutjnl-2012-304351 PMID: 23958557
17. Lee CW, Kuo WL, Yu MC, Chen TC, Tsai CN, Lee WC, et al. The expression of cytokeratin 19 in lymph nodes was a poor prognostic factor for hepatocellular carcinoma after hepatic resection. World J Surg Oncol. 2013; 11: 136. doi: 10.1186/1477-7819-11-136 PMID: 23758804

18. Ning S, Bin C, Na H, Peng S, Yi D, Xiang-hua Y, et al. Glypican-3, a novel prognostic marker of hepatocellular cancer, is related with postoperative metastasis and recurrence in hepatocellular cancer patients. Mol Biol Rep. 2012; 39: 351–357. doi: 10.1007/s11033-011-0745-y PMID: 21655958

19. Shirakawa H, Suzuki H, Shimomura M, Kojima M, Gotohda N, Takahashi S, et al. Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci. 2009; 100: 1403–1407. doi: 10.1111/j.1349-7006.2009.01206.x PMID: 19496787

20. Bosman FT, Carneiro F, Hruban RH, Theise ND. WHO classification of tumours of the digestive system. 4th ed. Lyon: World Health Organization; 2010: 205–227.

21. Sabattini E, Bisaard K, Ascani S, Poggi S, Piccioli M, Ceccarelli C, et al. The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. J Clin Pathol. 1998; 51: 506–511. PMID: 9797726

22. Fuji T, Zen Y, Harada K, Niwa H, Masuda S, Kaizaki Y, et al. Participation of liver cancer stem/progenitor cells in tumorigenesis of scirrhous hepatocellular carcinoma—human and cell culture study. Hum Pathol. 2008; 39: 1185–1196. doi: 10.1016/j.humpath.2007.12.010 PMID: 18547618

23. Pang A, Ng IO, Fan ST, Kwong YL. Clinicopathologic significance of genetic alterations in hepatocellular carcinoma. Cancer Genet Cytoenet. 2003; 146: 8–15. PMID: 14499690

24. Piao Z, Kim H, Jeon BK, Lee WJ, Park C. Relationship between loss of heterozygosity of tumor suppressor genes and histologic differentiation in hepatocellular carcinoma. Cancer. 1997; 80: 865–872. PMID: 9307185

25. Wong CM, Lee JM, Lau TC, Fan ST, Ng IO. Clinicopathological significance of loss of heterozygosity on chromosome 13q in hepatocellular carcinoma. Clin Cancer Res. 2002; 8: 2266–2272. PMID: 12114430

26. Khavari DA, Sen GL, Rinn JL. DNA methylation and epigenetic control of cellular differentiation. Cell Cycle. 2010; 9: 3880–3883. PMID: 20890116

27. Nishino K, Toyoda M, Yamazaki-Inoue M, Fukawatase Y, Chikazawa E, Sakaguchi H, et al. DNA methylation dynamics in human induced pluripotent stem cells over time. PLoS Genet. 2011; 7: e1002085. doi: 10.1371/journal.pgen.1002085 PMID: 21637780

28. Shen J, Wang S, Zhang YJ, Kappil M, Wu HC, Kibriya MG, et al. Genome-wide DNA methylation profiles in hepatocellular carcinoma. Hepatology. 2012; 55: 1799–1808. doi: 10.1002/hep.25569 PMID: 22234943

29. Wang XO, Ng RK, Ming X, Zhang W, Chen L, Chu AC, et al. Epigenetic regulation of pluripotent genes mediates stem cell features in human hepatocellular carcinoma and cancer cell lines. PLoS One. 2013; 8: e72435. doi: 10.1371/journal.pone.0072435 PMID: 24023739

30. Rumbajan JM, Maeda T, Souzaki R, Mitsui K, Higashimoto K, Nakabayashi K, et al. Comprehensive analyses of imprinted differentially methylated regions reveal epigenetic and genetic characteristics in hepatoblastoma. BMC Cancer. 2013; 13: 608. doi: 10.1186/1471-2407-13-608 PMID: 24373183

31. Chan AC, Fan ST, Poon RT, Cheung TT, Chok KS, Chan SC, et al. Evaluation of the seventh edition of the American Joint Committee on Cancer tumour-node-metastasis (TNM) staging system for patients undergoing curative resection of hepatocellular carcinoma: implications for the development of a refined staging system. HPB (Oxford). 2013; 15: 439–448. doi: 10.1111/hup.12477 PMID: 23776452

32. Feo F, Pascale RM. Multifocal hepatocellular carcinoma: intrahepatic metastasis or multicentric carcinoma? Ann Transl Med. 2015; 3: 4. doi: 10.3978/j.issn.2305-5839.2014.12.08 PMID: 25705636

33. Wang B, Xia CY, Lau WY, Lu XY, Dong H, Yu WL, et al. Determination of clonal origin of recurrent hepatocellular carcinoma for personalized therapy and outcomes evaluation: a new strategy for hepatic surgery. J Am Coll Surg. 2013; 217: 1054–1062. doi: 10.1016/j.jamcollsurg.2013.07.402 PMID: 24246620

34. Ramaswamy S, Tamayo P, Rifkin R, Mukherjee S, Yeang CH, Angelo M, et al. Multiclass cancer diagnosis using tumor gene expression signatures. Proc Natl Acad Sci U S A. 2001; 98: 15149–15154. doi: 10.1073/pnas.211566398 PMID: 11742071

35. Gong J, He XX, Tian A. Emerging role of microRNA in hepatocellular carcinoma (Review). Oncol Lett. 2015; 9: 1027–1033. doi: 10.3892/ol.2014.2816 PMID: 25663852

36. Mehta S, Shelling A, Muthukaruppan A, Lasham A, Blenkiron C, Laking G, et al. Predictive and prognostic molecular markers for cancer medicine. Ther Adv Med Oncol. 2010; 2: 125–148. doi: 10.1177/1758834009360519 PMID: 21789130

37. Shibata T, Aburatani H. Exploration of liver cancer genomes. Nat Rev Gastroenterol Hepatol. 2014; 11: 340–349. doi: 10.1038/nrgastro.2014.6 PMID: 24473361