Stem cell factor is a novel independent prognostic biomarker for hepatocellular carcinoma after curative resection

Xiuchao Wang1, He Ren1 Tiansuo Zhao, Jing Chen, Wei Sun, Yan Sun1, Weidong Ma, Jian Wang, Chuntao Gao, Song Gao, Mingxiao Li, Jia2 and Jihui Hao*

Department of Abdominal Oncology and 1Department of Pathology, National Clinical Research Center for Cancer, Key Lab of Cancer Treatment and Prevention, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, China and 2Centre for Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, London, EC1M 6BQ, UK

*To whom correspondence should be addressed. Tel: +86 2223340123 3070; Fax: +86 2223537796; Email: haojihui@tjmuch.com
Correspondence may also be addressed to Li Jia. Tel: +86 (0)20 7882 3825; Fax: +44 (0)20 7882 3891; Email: l.jia@qmul.ac.uk

Stem cell factor (SCF), a ligand of c-kit, is a hematopoietic growth factor. Uncontrolled activity of SCF/c-kit signaling pathway contributes to the formation of a variety of human malignancies. In this study, we determined whether SCF expression could risk-stratify patients with hepatocellular carcinoma (HCC) after curative resection. HCC tissues from 160 patients were collected during curative resection and stained with SCF and CD34, a marker for microvessel density (MVD), using immunohistochemistry. Two statistical analyses were performed: an independent continuous and a multivariate categorical analysis, with test/validation set-defined cut points, and Kaplan–Meier estimated outcome measures of overall survival (OS) and relapse-free survival (RFS). We found that higher levels of SCF confer worse OS (continuous P = 0.014; and categorical P = 0.009), and RFS (continuous P = 0.002; categorical P = 0.003) of patients with HCC. SCF varies independently from MVD-CD34, tumor node metastasis, histologic grade, age and gender, and retains prognostic significance when analysed as a categorical variable in a multivariate analysis. We confirmed that MVD-CD34 is also an independent prognostic marker for patients with HCC. The levels of SCF and CD34 showed a positive and significant correlation (P < 0.0001) and double low expression confers superior OS (median = 48 months) and RFS (median = 24 months), whereas double high expression confers shortest RFS (median = 10.5 months) compared with single measurements. The prognostic values of SCF and CD34 were independently determined in this study and we propose that both of them are independent prognostic markers for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality worldwide, and the second leading cause of cancer deaths in the world (1–3). Chronic hepatitis B or hepatitis C, aflatoxin B exposure and alcoholism are the leading risk factors for HCC (4–7). Although immediate results of hepatic resection for HCC have been greatly improved, the long-term prognosis after resection of HCC remains unsatisfactory as a result of a high incidence of recurrence. Prevention and effective management of recurrence are the most important strategies to improve the long-term survival rates (8–10).

Stem cell factor (SCF), also called c-kit ligand) is a dimeric molecule that exerts its biological functions by binding to and activating the receptor tyrosine kinase c-Kit. SCF-mediated c-Kit signaling pathway plays important roles in mediating angiogenesis, migration, cell survival and proliferation (11,12). Uncontrolled activity of c-Kit contributes to the formation of a variety of human malignancies (12,13). The morphological and immunohistochemical analysis revealed that several human tumor tissues, such as human cervical adenocarcinoma carcinomas (14), Merkel cell carcinoma (15), renal tumor (16) and rat cholangiocarcinogenesis (17) simultaneously express both SCF and c-kit proteins. It was recently reported that co-stimulation with SCF and erythropoietin induces migration of c-kit expression in cervical cancer cells through the sustained activation of ERK1/2 (18). HCC expressing ‘stemness’-related markers, such as c-Kit and CD34, have been associated with aggressive biological behavior and poor prognosis (19,20). Expression of c-kit protein was detected in 70% of human HCC and 90% of the corresponding peritumoral non-cirrhotic, as well as in cirrhotic liver tissues (21). However, expression of SCF in human HCC tissue and its impact on the clinical outcomes of patients with HCC have not been previously reported.

The tumor microenvironment plays important roles during cancer evolution and metastasis (22–25). In particular the formation of hypoxic areas within the expanding mass of a solid tumor and the subsequent induction of an angiogenic switch are crucial steps that shape tumor progression (24,26,27). As a consequence, the extensive transcriptional response regulates angiogenesis, glucose metabolism, cell growth and metastasis process (28). Angiogenesis is a critical step in the development and progression of HCC (29) and is strongly associated with an increased risk of recurrence, and a shorter recurrence time after resection of HCC (23,30–32). Increasing density of newly formed microvessels in growing tumors correlates closely with an increasing number of tumor cells shed into the bloodstream. Microvessel density (MVD) is the most recognized indicator to evaluate angiogenesis of solid tumors, and quantitation of intratumor MVD by immunohistochemistry staining for vascular endothelial cell markers, such as CD34, has been proved as a useful prognostic predictor in HCC patients (33). Although the auto-crine SCF/c-kit signaling plays an important role in mediating the pro-angiogenic effects of human umbilical vein endothelial cells (34), the role of SCF/c-Kit pathway in HCC has not been previously reported.

SCF and c-kit receptors are widely expressed during normal embryonic development, and their expression in the adult is limited (35). It is unknown whether SCF is expressed in HCC tissue. This study aims to determine whether SCF as well as MVD-CD34 can be used as independent and reliable prognostic biomarkers for HCC patients after curative resection.

Materials and methods

Human samples and ethical considerations

Tissue specimens were obtained through the Tumor Tissue Bank of Tianjin Cancer Institute from 160 patients, who underwent curative resection for HCC at the Tianjin Cancer Institute and Hospital between 2008 and 2012. Ethical approval for this study was obtained from the local ethics committee. Patients gave verbal consent for the use of their tumor tissues for future investigations, which had been performed for many years at time of the initial diagnosis. The diagnoses of these HCC samples were verified by senior pathologists. None of the patients had received chemotherapy or radiotherapy at the time the tissue samples were taken. Detailed pathologic and clinical data were collected for all samples, including the Edmondson tumor grade, TNM stage and α-fetoprotein (AFP) values (Supplementary Table 1, available at Carcinogenesis Online). The median follow-up time for overall survival (OS) and relapse-free survival (RFS) were 36 and 18 months, respectively, for patients at the time of analysis (Supplementary Figure 1 and Table 2, available at Carcinogenesis Online). In addition, 30 normal liver tissues were obtained from adult patients who underwent hepatectomy for benign conditions rather than liver malignancies.

Follow-up and diagnosis of tumor recurrence

Post-operative patients were followed-up periodically to exclude recurrence of HCC. Biochemical liver function tests, serum AFP levels and abdominal

Abbreviations: AFP, α-fetoprotein; CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio; MVD, microvessel density; OS, overall survival; RFS, relapse-free survival; SCF, stem cell factor.

1These authors contributed equally to this work.
Fig. 1. SCF expression and clinical outcomes. (A) Representative immunohistochemical analysis of SCF expression in normal liver and HCC samples. Images were taken at two different magnifications (×100 and ×400). (B) Overall expression of SCF. The levels of SCF expression were presented as % of SCF positive cells. Data presented are medians with interquartile ranges: 5% (0–10%) for normal and 54% (20–80%) for HCC tissues, respectively. The significant difference in SCF expression between HCC and normal liver tissues were analysed by the Mann–Whitney U test. (C and D) Kaplan–Meier survival curves of HCC patients after surgery for HCC according to SCF expression were generated with GraphPad Prism program. (C) OS of HCC patients was based on low expression ≤70% and high expression >70%. (D) RFS of HCC patients was based on low expression ≤85% and high expression >85%. Cut points were generated by the X-Tile software.

Table I. Univariate analysis of continuous covariates

<table>
<thead>
<tr>
<th>Covariates</th>
<th>OS</th>
<th></th>
<th>RFS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>SCF</td>
<td>1.010 (1.002–1.017)</td>
<td>0.014</td>
<td>1.010 (1.003–1.016)</td>
<td>0.002</td>
</tr>
<tr>
<td>CD34</td>
<td>1.016 (1.001–1.031)</td>
<td>0.036</td>
<td>1.019 (1.008–1.030)</td>
<td>0.001</td>
</tr>
<tr>
<td>TNM</td>
<td>1.595 (1.246–2.043)</td>
<td>&lt;0.0001</td>
<td>1.544 (1.277–1.866)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tumor size</td>
<td>1.098 (1.046–1.153)</td>
<td>&lt;0.0001</td>
<td>1.110 (1.066–1.156)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AFP</td>
<td>1.000 (1.000–1.000)</td>
<td>0.003</td>
<td>1.000 (1.000–1.000)</td>
<td>0.031</td>
</tr>
<tr>
<td>Age</td>
<td>1.010 (0.982–1.039)</td>
<td>0.484</td>
<td>1.005 (0.983–1.026)</td>
<td>0.678</td>
</tr>
</tbody>
</table>

TNM, TNM classification of malignant tumors (I, II, III and IV); tumor size: centimeters; AFP (0.75-240761); age, from 29 to 79. Data shown were analysed by Cox proportional hazard model using SPSS.
ultrasoundography were performed every 3 months after resection. Update inquiries about current survival status of all patients were made by telephone calls by the end of March 2014. Recurrence was diagnosed on the basis of two-photon coincidence imaging technology, or the combination of increased serum AFP levels with consistent findings of ultrasonography, computed tomography or magnetic resonance imaging, according to the latest guidelines released.

**Immunohistochemistry**

HCC specimens were formalin fixed, paraffin embedded and sectioned (4 μm in thickness). The slides were deparaffinized in xylene and rehydrated through graded ethanol to water before staining. All sections were treated with 5 mM citrate buffer (pH 6.0) for antigen retrieval and with 3% H₂O₂ for the inactivation of endogenous peroxidase. After blocking for 30 min, sections were incubated with anti-SCF (Abcam, ab8021, at 1:350 dilution) or anti-CD34 (Santa Cruz Biotechnology, sc-133082, at 1:150 dilution) antibodies overnight at 4°C. After wash, the sections were stained with a secondary antibody for 30 min at room temperature. Diaminobenzidine and hematoxylin were used as a chromogen substrate and for nuclear counterstaining, respectively. Phosphate-buffered saline was substituted for each primary antibody as a negative control. Five random fields (×400) were examined under a light microscope. Evaluation of immunohistochemistry staining results was blindly and independently performed by two pathologists who were blinded to the clinical data. In the tissue cores with mixtures of tumor cells and stromal cells, the intensity of tumor cells was scored according to morphological criteria. Levels of SCF expression was calculated as percentage of positive cells.

MVD-CD34 was determined in vascular ‘hot spots’, the mean microvessel count of the five most vascular areas was taken as the MVD, which was expressed as the absolute number of microvessels per 0.74 mm² (×200). Any cluster of endothelial cells with CD34 positive was counted as a single microvessel (33). To reduce observer-related variation, the counting of the microvessels was performed using a computer image analyser. An automated microvessel count/field was computed in each hot spot, and the mean microvessel numbers of three most vascularized areas were taken as the levels of MVD-CD34.

**Statistical analysis**

Statistical analysis was performed using IBM SPSS version 22 for Windows and GraphPad Prism version 5.0.3. Data are shown as median with interquartile range due to high variation among samples. Significant differences between groups with unequal size were analysed with the Mann–Whitney U test. Pearson product-moment correlation method was used to analyse linear correlation between two groups. Fisher’s exact test (contingency table) was used to analyse frequency distribution between two categorical variables. Two independent statistical analyses, continuous and categorical data analysis were conducted to increase the robustness of statistical inferences. The continuous data analysis evaluates the prognostic effect of each of these biomarkers treated as continuous variables using Cox regression analysis. The categorical data analysis was a clinically applicable method, dividing patients categorically according to the expression of each biomarker into cohorts, on the basis of levels of expression (36). Outcomes, measured from date of resection to occurrence of event or date of last follow-up, were OS and RFS, the event being relapse or recurrent after curative resection. All P values less than 0.05 were considered statistically significant. *, ** and *** indicate P value < 0.05, 0.001 and 0.0001, respectively (36,37).

COX proportional hazards models were used for continuous data analysis to generate hazard ratio (HR) with 95% confidence intervals (CI) for SCF, CD34 and other established clinical risk factors for each OS and RFS. Categorical (cut point) data analysis was performed using the X-tile statistical package (Yale University, New Haven, CT) (38) enabling cut points to be determined for markers without validated normal ranges. The X-tile software divides the cohort into two independent data sets (a test and a validation sets) in a 1:2 ratio, determines optimal cut points for each marker for the test set, and applies to the validation set (36). Kaplan–Meier survival curves were made according to these cut points and P values, HR and 95% CI were generated by log-rank (Mantel–Cox) test. Univariate analyses were performed first, including one factor a time to predict their effects on the clinical outcome. Multivariate analysis was performed using a Cox proportional hazards model to assess the independent effect of prognostic variables on the outcome. Relevant factors were considered simultaneously through the use of both forward and backward stepwise models (39). To determine whether SCF or CD34 causes confounding, two categorical variables were adjusted or stratified with each other and the estimated measures of association before (HR_{adjusted}) and after adjusting (HR_{adjusted}) for confounding were calculated as: magnitude of confounding = (HR_{adjusted} - HR_{unadjusted})/HR_{unadjusted} × 100%. If the difference between the two measures of association is 10% or more, then confounding was present (http://sphweb.bumc.bu.edu).

**Results**

**Significantly increased expression of stem cell factor in hepatocellular carcinoma**

To distinguish the origin of SCF in the liver, expression of the intra-cellular SCF was determined in 160 HCC and 30 normal adult liver tissues using immunohistochemistry staining and the results were reviewed by expert histopathologists, suggesting that SCF is expressed by hepatocytes in both HCC and normal samples (Figure 1A). SCF protein was detected in either the cytoplasm or the nucleus. The levels of SCF were expressed as % of SCF positive cells. Normal liver tissue sections of increased serum AFP levels with consistent findings of ultrasonography, or the combination of increased serum AFP levels with consistent findings of ultrasonography, computed tomography or magnetic resonance imaging were excluded for the selection.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Covariates (cut points)</th>
<th>OS/RFS months†</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>SCP (&gt;70 versus ≤70)</td>
<td>29:40</td>
<td>1.895 (1.176–3.046)</td>
<td>1.826 (1.113–2.995)</td>
</tr>
<tr>
<td></td>
<td>Gender (F versus M)</td>
<td>21:36</td>
<td>3.557 (1.652–7.658)</td>
<td>2.283 (1.271–4.101)</td>
</tr>
<tr>
<td></td>
<td>Age² (&gt;62 versus ≤62)</td>
<td>36:34</td>
<td>1.736 (0.863–3.502)</td>
<td>2.191 (1.172–4.095)</td>
</tr>
<tr>
<td></td>
<td>TNM (III–IV versus I–II)</td>
<td>21:40</td>
<td>3.414 (1.863–6.256)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Grade² (3:2:1)</td>
<td>20:35:48</td>
<td>2.026 (1.477–2.788)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor size³ (&gt;3.5 cm versus ≤3.5 cm)</td>
<td>30:45</td>
<td>2.173 (1.337–5.350)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Vascular invasion (pos. versus neg.)</td>
<td>36:36</td>
<td>2.036 (1.183–3.503)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>HBV (pos. versus neg.)</td>
<td>36:36</td>
<td>1.032 (0.588–1.781)</td>
<td>0.9536</td>
</tr>
<tr>
<td></td>
<td>AFP² (&gt;160 versus ≤160 U/L)</td>
<td>31:40</td>
<td>1.628 (1.006–2.634)</td>
<td>0.0472</td>
</tr>
<tr>
<td>RFS</td>
<td>SCP (&gt;85 versus ≤85)</td>
<td>12:21</td>
<td>1.965 (1.260–3.064)</td>
<td>2.053 (1.291–3.263)</td>
</tr>
<tr>
<td></td>
<td>Gender (F versus M)</td>
<td>14:20</td>
<td>2.041 (1.126–3.908)</td>
<td>1.677 (1.018–2.764)</td>
</tr>
<tr>
<td></td>
<td>Age² (&gt;64 versus ≤64)</td>
<td>12:21</td>
<td>3.684 (1.846–7.535)</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>TNM (III–IV versus I–II)</td>
<td>12:27</td>
<td>2.462 (1.548–3.915)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Grade² (3:2:1)</td>
<td>20:18:30</td>
<td>1.532 (1.209–1.941)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor size³ (&gt;5 cm versus ≤5 cm)</td>
<td>12:28</td>
<td>2.281 (1.504–3.461)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Vascular invasion (pos. versus neg.)</td>
<td>12:28</td>
<td>3.590 (2.305–5.593)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>HBV (pos. versus neg.)</td>
<td>18:21</td>
<td>1.172 (0.769–1.789)</td>
<td>0.4612</td>
</tr>
<tr>
<td></td>
<td>AFP² (&gt;4000 versus ≤4000 U/L)</td>
<td>10:21</td>
<td>4.414 (1.942–10.03)</td>
<td>&lt;0.0004</td>
</tr>
</tbody>
</table>

Covariates that were included in the multivariate analysis were first selected using the forward and then the backward stepwise.

†Calculated microvessel count/field was computed in each hot spot, and the mean microvessel numbers of three most vascularized areas were taken as the levels of MVD-CD34.

**Statistical analysis**

Significant differences between groups with unequal size were analysed with the Mann–Whitney U test. Pearson product-moment correlation method was used to analyse linear correlation between two groups. Fisher’s exact test (contingency table) was used to analyse frequency distribution between two categorical variables. Two independent statistical analyses, continuous and categorical data analysis were conducted to increase the robustness of statistical inferences. The continuous data analysis evaluates the prognostic effect of each of these biomarkers treated as continuous variables using Cox regression analysis. The categorical data analysis was a clinically applicable method, dividing patients categorically according to the expression of each biomarker into cohorts, on the basis of levels of expression (36). Outcomes, measured from date of resection to occurrence of event or date of last follow-up, were OS and RFS, the event being relapse or recurrent after curative resection. All P values less than 0.05 were considered statistically significant. *, ** and *** indicate P value < 0.05, 0.001 and 0.0001, respectively (36,37).

Results

Significantly increased expression of stem cell factor in hepatocellular carcinoma

To distinguish the origin of SCF in the liver, expression of the intra-cellular SCF was determined in 160 HCC and 30 normal adult liver tissues using immunohistochemistry staining and the results were reviewed by expert histopathologists, suggesting that SCF is expressed by hepatocytes in both HCC and normal samples (Figure 1A). SCF protein was detected in either the cytoplasm or the nucleus. The levels of SCF were expressed as % of SCF positive cells. Normal liver...
cells showed either very low or no SCF expression. In contrast, SCF expression in HCC samples displayed a heterogeneous expression pattern and the median levels (54%) were significantly higher than in the normal controls (5%; \(P < 0.0001\); Figure 1B). Thus, we report, for the first time, that human HCC cells express higher levels of SCF.

**Stem cell factor is an independent prognostic biomarker for the clinical outcomes of hepatocellular carcinoma patients**

The prognostic significance of SCF on the clinical outcomes of HCC patients was first determined using continuous data analysis method and compared with other risk factors. SCF expression showed significant impact on OS (\(P = 0.014\)) and RFS (\(P = 0.002\)). Other risk factors (continuous variables), such as TNM, tumor size and AFP also showed prognostic significance (Table I). However, the age did not show prognostic significance when continuous data were used for the analysis. These results demonstrate that SCF as well as other risk factor are independent prognostic biomarkers for predicting the outcomes of HCC patients.

Using categorical data analysis, two prognostic groups with low and high expression of SCF, and other continuous variables such as age, tumor size and AFP value, were defined using the X-Tile software to generate cut point for each outcome. TNM stages were also categorized as low (I and II) and high (III-IV) groups (Table II). Results showed that higher expression of SCF was significantly associated shorter OS (\(P = 0.009\); HR = 1.90) and RFS (\(P = 0.003\); HR = 1.97; Figure 1C and D; Table II). Other categorical risk factors, such as gender, TNM (III-IV versus I-II), tumor grade (3:2:1), tumor size (large versus small), vascular invasion (VI; positive versus negative), AFP (high versus low) all showed prognostic significance on both.

![Fig. 2. MVD-CD34 expression and clinical outcomes](https://academic.oup.com/carcin/article-abstract/35/10/2283/324130) by guest on 01 May 2019

**Fig. 2.** MVD-CD34 expression and clinical outcomes. (A) Representative immunohistochemical analysis of MVD-CD34 expression in normal liver and HCC samples. (B) Overall expression of MDV-CD34. Data presented are medians with interquartile ranges: 7.17 (3.25–8.33) for normal and 23.33 (11.08–35.33) for HCC tissues, respectively. The significant difference in CD34 expression between HCC and normal liver tissues were analysed by the Mann–Whitney \(U\) test. (C and D) Kaplan–Meier survival curves of HCC patients after surgery for HCC according to CD34 expression were generated with GraphPad Prism program. (C) OS of HCC patients was based on low expression 30.67 and high expression >30.67. (D) RFS of HCC patients was based on low expression 26.33 and high expression >26.33. Cut points were generated by the X-Tile software.
OS and RFS (Table II; Supplementary Figures 2 and 3, available at Carcinogenesis Online). However, age showed significant prognosis only on RFS but not OS (Supplementary Figure 4 and Table 2, available at Carcinogenesis Online). HBV infection did not show any prognostic significance.

For determining the confounding effect of SCF on other risk factors, multivariate analysis was first conducted by combination of SCF with each covariable (risk factor) one by one. Although the confounding effect exists between SCF and other risk factor, the prognostic significance of SCF and other risk factors on both OS and RFS retained after adjusting with each other. However, SCF lost its statistical prognostic significance on RFS (HR = 0.058; 95% CI = 0.99–2.45) only after adjusted by the risk factor, vascular invasion (Supplementary Table 3, available at Carcinogenesis Online). The association of SCF with multiple risk factors on the clinical outcomes was also evaluated by both forward and backward stepwise selections. The prognostic significance of SCF on OS remained when combined with gender, age and TNM stages. Importantly, this combination increased the statistical power of age on OS, the P value decreased from 0.121 to 0.014 (Table II). Similarly, SCF retained prognostic significance on RFS when combined with gender, age, TNM and grade (Table II). Tumor size and vascular invasion were not included in these statistical combinations because they are integrated components of TNM. These results demonstrate that SCF is an independent prognostic biomarker and is strongly associated with increased risk of HCC.

**MVD-CD34 is an independent prognostic biomarker for the clinical outcomes of hepatocellular carcinoma patients**

It was previously reported that high MVD-CD34 is a predictive biomarker of early post-resection of recurrence in patients with HCC (40). MVD-CD34 was determined in this cohort of HCC samples by immunohistochemistry staining and the results were reviewed by histopathologists (Figure 2A). HCC samples showed significantly increased MVD-CD34 levels (median = 23.3) compared with the normal liver samples (median = 7.2; P < 0.0001; Figure 2B). MVD-CD34 as an independent prognostic factor for OS (P = 0.036) and RFS (P = 0.001) in patients with HCC was proved using the univariate analysis (Table I). The categorical analysis showed that HCC patients with high MVD-CD34 had significantly shorter OS (HR = 0.002; HR = 2.14) and RFS (HR = 0.001; HR = 1.88; Figure 2C and D; Table III).

Confounding effect of CD34 on other HCC risk factors were tested by multivariate analysis using the Cox regression model. When CD34 was adjusted by each covariate one by one, CD34 showed confounding effect on other risk factors for both OS and RFS. Nevertheless, their prognostic significance remained after being adjusted with each other (Supplementary Tables 5 and 6, available at Carcinogenesis Online). The associations of MVD-CD34 with multiple risk factors were evaluated by the backward stepwise model. The MVD-CD34 remained a similar statistical power (P = 0.006; HR = 2.06) on OS when adjusting for gender, age and AFP compared with the unadjusted measure (P = 0.002; HR = 2.14). The statistical power of CD34 on RFS remained unchanged after being adjusting for age, gender, TNM and grade together (P = 0.001; HR = 1.903) compared with the prognostic effect of unadjusted CD34 on RFS (HR = 0.001; HR = 1.879; Table III). We demonstrate that MVD-CD34 alone is also an independent prognostic biomarker and strongly associated with increased risk for this cohort of HCC patients.

**Stem cell factor expression is positively correlated with MVD-CD34**

We next determined the correlation between SCF and CD34 at their protein expression levels. Linear correlation was used to determine the relationship between SCF and CD34 expression, as two continuous variables. The levels of SCF expression were significantly correlated with those of CD34 (P < 0.0001; r = 0.4155; Supplementary Figure 5A, available at Carcinogenesis Online). The contingency tables (Supplementary Figure 5B and C, available at Carcinogenesis Online) were employed to determine the frequency distribution of both SCF and CD34, as two categorical variables. The cut points between negative and positive expression of SCF and CD34 were defined previously for OS and RFS using the X-tile software (Tables II and III). The frequency distribution results further demonstrate that there was a strong association between SCF and CD34 expression (P < 0.0001), as analysed by the Fisher’s exact test.

**Lower expression of both stem cell factor and MVD-CD34 is associated with favorable prognosis**

As it was shown above, SCF and CD34 both are independent risk factors for HCC and their expression levels were significantly correlated with each other. We then tested the association between SCF and CD34 on the clinical outcomes. When the prognostic effect of CD34 on OS was stratified by SCF, it caused 12.7% confounding effect and the P value changed from 0.002 to 0.021. This indicates that CD34
remains its prognostic significance in the presence of SCF. In contrast, when stratified with CD34, the prognostic significance of SCF was affected by CD34, 16.1% for OS and 12.8% for RFS and the P values changed from 0.009 to 0.074 for OS and from 0.003 to 0.052 for RFS (Table III). In the presence of CD34, SCF is no longer a significant prognostic marker for the clinical outcomes of HCC patients. This indicates that SCF and CD34 do not have causal relationship, although two variables are biologically correlated.

To further determine the association between SCF and CD34 on the clinical outcomes in HCC, we combined SCF and CD34 variables and categorized them into four subgroups: i.e. SCF<sub>LOW</sub>/CD34<sub>LOW</sub>, SCF<sub>HIGH</sub>/CD34<sub>HIGH</sub>, SCF<sub>LOW</sub>/CD34<sub>HIGH</sub> and SCF<sub>LOW</sub>/CD34<sub>LOW</sub> (Figure 3A and B). The prognostic significance of these subgroups was evaluated by multivariate analysis. The SCF<sub>LOW</sub>/CD34<sub>LOW</sub> subgroup showed longest survival rates for both OS (median = 48 months) and RFS (median = 24 months) and therefore was used as a reference. Other three subgroups showed significantly decreased OS rates compared with the SCF<sub>LOW</sub>/CD34<sub>LOW</sub> subgroup. The SCF<sub>HIGH</sub>/CD34<sub>HIGH</sub>, SCF<sub>LOW</sub>/CD34<sub>HIGH</sub> and SCF<sub>HIGH</sub>/CD34<sub>LOW</sub> subgroups showed significantly shortened OS compared with the SCF<sub>LOW</sub>/CD34<sub>LOW</sub> subgroup, confirming that higher expression of either SCF or CD34 confers a poor OS. However, the SCF<sub>HIGH</sub>/CD34<sub>HIGH</sub> subgroup showed shortest RFS rate (median = 10.5 months; P < 0.0001; HR = 3.32) compared with the SCF<sub>LOW</sub>/CD34<sub>LOW</sub> subgroup (median = 24 months). However, the SCF<sub>HIGH</sub>/CD34<sub>LOW</sub> subgroup did not show prognostic significance for RFS (Table III and Figure 3A and B). These results propose that lower expression of both SCF and CD34 is associated with favorable clinical outcome in patients with HCC. In contrast, higher expression of both SCF and CD34 predicts the shortest time to recurrence of patients with HCC.

Discussion

The results of the continuous and multivariate categorical statistical analyses present, for the first time, reliable evidence that SCF is a novel, independent prognostic biomarker for overall and relapse-free survival of patients with HCC. The median time to recurrence is 12 months for patients with SCF expression > 85% and 10.5 months when concurrent with CD34 expression > 26.33% (15% of patients) compared with 24 months for patients with lower expression of both proteins. Lower expression of both SCF and CD34 confers favorable prognosis and the median overall survival rate is 48 months which is higher than the average of 36 months in this cohort. Currently, AFP is the only serum biomarker that has widely been used to screen and diagnose HCC in China (41). Our data showed that the median to recurrence is 10 months for HCC patients who serum AFP levels were >4000 U/l and about 9% of HCC patients reached this level. The AFP levels in 50% HCC patients were ≤200 U/l. Therefore, AFP has its limitation in predicting the recurrence of patients with HCC after operation. Although the large number of studies dedicated to molecular diagnosis of HCC, truly reliable biomarkers for this neoplasm still need to be identified (42,43). To the best of knowledge, this study demonstrates for the first time that SCF is a reliable and independent prognosis biomarker for predicting recurrence for HCC patients after curative resection.

SCF, also known as c-kit ligand, mast cell growth factor or steel factor, is a hematopoietic cytokine that triggers its biologic effects by binding to its receptor, expressing the c-kit proto-oncogene (44,45). It was detected that c-Kit was detected in liver with intermediate carcinomas, transitional CHCs and HCC small cell type (46). Although it is constitutively produced by endothelial cells and fibroblasts, SCF was initially isolated from the medium conditioned by Buffalo rat liver cells (45). It was detected that SCF and c-kit receptor are widely expressed during normal embryonic development and their expression in the adult is limited. However, both SCF and c-kit were expressed in liver putative stem cells in adult rat during liver regeneration, suggesting that the SCF/c-kit system may be involved in the early activation of the hepatic stem cells as well as in the expansion and differentiation of oval cells (35). It was recently found that isolated HCC progenitor cells from different mouse HCC models give rise to cancer when they were introduced into a liver undergoing chronic damage and compensatory proliferation (47). HCC expressing 'stemness'-related proteins are characterized by increased telomere length and chromosomal instability compared with conventional HCC (20). These evidences suggest that SCF expressed in HCC may play an important role in tumorigenesis and progression.

To validate whether SCF is an independent prognostic biomarker for HCC, we first determined the prognostic significance using continuous data analysis. Similar to other established risk factors, such as TNM, tumor grade, tumor size and AFP, SCF showed significantly prognostic for both OS and RFS, independent of other risk factors. We then categorized HCC patients with SCF high and SCF low groups according to the X-tile software. The categorized analysis confirmed that higher SCF expression was strongly associated with shorter OS and RFS. The association between SCF and other risk factors were
Biomarker for hepatocellular carcinoma

detected by using the multivariate analysis. The prognostic significance of SCF retained after adjusted for single or multiple risk factors, regardless of the confounding effects between each other. This further confirmed that SCF is a reliable prognostic biomarker to predict the clinical outcomes, independent of other risk factors.

Similar to SCF, CD34 is also expressed in hematopoietic cells and non-hematopoietic progenitor, adult stem cells and endothelial cells of blood vessels (48). CD34 has been used as a marker for MVD which is involved in the dismal prognosis of HCC (30). Indeed, we confirmed that CD34-MVD is also an independent prognostic biomarker for HCC, CD34 and SCF showed the strongest correlation and poor prognosis of HCC (19). We then sought to determine whether increased expression of SCF could be a potential cause of tumor angiogenesis in HCC. The levels of SCF and CD34 expression were strongly correlated with each other as determined by both Pearson’s correlation and the Fisher’s exact test. However, the multivariate analysis showed that CD34 is a confounder of SCF which lost prognostic significance after adjusted by CD34. This suggests that SCF does not have a causal relationship with CD34; at least it may not be a direct cause of angiogenesis of HCC.

In conclusion, we demonstrate that SCF is expressed in HCC tissue and higher expression of SCF is associated with poor prognosis of patients with HCC. Although no causal relationship between SCF and CD34, patients with double positive expression of SCF and CD34 showed the shortest time to recurrence. In spite of the reliable prognostic value of SCF, we also propose that SCF could be a potential therapeutic target for the treatment of HCC.

Supplementary material

Supplementary Figures 1–5 and Supplementary Tables 1–6 can be found at http://carcin.oxfordjournals.org/

Funding

National Natural Science Foundation of China (81302082, 81272685, 3130151 and 81172355); Major Anticancer Technologies R&D Program of Tianjin (11JCZDJC18400); Key Program of National Natural Science Foundation of Tianjin (11JCZDJC18400 and 13YCYBYC37400).

Acknowledgements

We thank L.Z. in the Department of Pathology, Tianjin Medical University Cancer Institute for providing hepatocellular carcinoma sections, and W.X. and J.L. for collecting clinical data.

Conflict of Interest Statement: None declared.

References


Received May 22, 2014; revised July 9, 2014; accepted July 25, 2014