Expression and clinical significance of the stem cell marker CD133 in hepatocellular carcinoma

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SUMMARY

Background: Although the primitive haematopoietic and neuronal stem cell marker CD133 is known to be present in cancer stem cells (CSCs) in hepatocellular carcinoma (HCC), the postresection prognostic impact of CD133 in HCC patients remains limited. Methods: Sixty-three resected specimens were collected from HCC patients. The expression of CD133 protein was analysed by immunohistochemistry and the association of CD133 expression with clinicopathological characteristics, tumour recurrence and survival of the patients was evaluated. Results: Immunohistochemical analysis of 63 HCC tissue specimens revealed that CD133 positive tumour cells were frequently present in HCC. Increased CD133 immunostaining was found in 26 specimens (41.3%). Increased CD133 expression levels were correlated with increased tumour grade, advanced disease stage, and elevated serum alpha-fetoprotein levels. Kaplan–Meier analysis indicated that patients with increased CD133 levels had shorter overall survival and higher recurrence rates compared with patients with low CD133 expression. Multivariate analyses revealed that increased CD133 expression was an independent prognostic factor for survival and tumour recurrence in patients with HCC. Conclusions: These findings suggest that reactivated CD133 positive cells are frequently present in HCC. Additionally, increased CD133 expression corresponds with higher stage tumours in HCC, thus indicating a poor prognosis for patients. These data support the CSC hypothesis.

Introduction

Cancer may be initiated and maintained by a rare fraction of cancer stem cells (CSCs) that are able to self-renew, differentiate and proliferate (1). The existence of CSCs is well documented in many different cancers, including acute myeloid leukaemia, breast cancer, brain cancer, prostate cancer and lung cancer (2–7). Furthermore, the CSC hypothesis asserts that CSCs have many properties of normal stem cells, and influence the prognosis of patients by promoting the recurrence, metastasis and multidrug resistance of tumours (8,9). Indeed, growing evidence supports this hypothesis, demonstrating that CSCs participate in carcinogenesis and may influence the prognosis of patients (10–13).

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in many countries worldwide. Despite great progress in diagnosis and treatment, the prognosis for patients with HCC is still unsatisfactory and unpredictable because of high rates of recurrence and metastasis (14–16). The precise molecular mechanisms involved in the initiation and progression of HCC are unclear. In HCC, morphologically diverse cells express a variety of hepatic lineage markers. In fact, many studies have shown that stem/progenitor cells are involved in hepatocarcinogenesis in certain rodent models and activated stem/progenitor cells are present in human HCC (17–21). Thus, isolation and identification of CSCs could elucidate the cellular origin of HCC tumour cells, providing insight into the molecular mechanisms of carcinogenesis.

CD133 (AC133) is a five transmembrane cell surface glycoprotein, originally identified in a subpopulation of CD34-positive haematopoietic stem cells derived from human fetal bone marrow, fetal liver or peripheral blood (22,23). CD133 is known to be present in endothelial stem cells, neuronal and glial stem cells, and renal and prostatic epithelial stem cells (24–26). Researchers have used CD133 to find CSCs in several malignant tumour tissues such as...
acute myeloid leukaemia, and brain and colon cancers (2,27,28) and found that CD133 expression is increased in many human malignancies and is a potential prognostic marker (29). More recently, a CD133-positive subpopulation of multipotent cells with extensive proliferative and self-renewal abilities was identified as CSCs in several HCC cell lines, and was proven to contribute to the initiation and growth of HCC (30,31) supporting the CSC hypothesis. Furthermore, re-expression of CD133 has been reported in regenerating rat liver (31,32) indicating that CD133 is associated with liver cell proliferation, a possible link to liver cancer.

CD133-positive CSCs could be involved in the pathogenesis of HCC. However, little is known about the relationship between CSCs and the clinical outcome of HCC, or the long-term prognostic impact of CSCs in HCC. Consequently, we investigated the expression of CD133 in HCC, and then examined the potential prognostic value of CD133 expression in patients with HCC.

Materials and methods

Patients and tissue specimens

A total of 63 HCC surgical resection specimens were collected at Xijing Hospital of the Fourth Military Medical University, Xi’an, China, from March 2000 to October 2001 with the patients’ consents. The patients included 52 men and 11 women with ages ranging from 24 to 76 years (mean: 50.3 ± 12.4 years). HCC was diagnosed by biochemical tests, sonograms and computed tomography (CT) scans, with histological confirmation. Tumour size was recorded as the greatest dimension of each specimen. Tumours were staged by using the International Union Against Cancer (UICC) 2002 issue of the TNM system (New York, NY, USA), while tumour grading was performed as outlined by Edmondson and Steiner (33). The clinicopathological features of the 63 HCC patients are listed in Table 1. Ten normal adult and four fetal liver specimens were collected as negative and positive controls respectively. The Ethics Committee of Fourth Military Medical University approved the study protocol.

Patients’ follow-up and prognosis

Only 60 patients were enrolled in the survival comparison, because three patients died as a result of complications or postsurgical hepatic failure. The patients had regular follow-up examinations. An alpha-fetoprotein (AFP) assay (monthly) and sonography or CT detected a tumour (tumours). Disease-free survival was calculated from the date of surgery to the date the tumour recurred, and overall survival was calculated from the date of surgery to death. The end-point for follow-up was set at 5 years, and the survival time was set at 60 months for those who survived more than 5 years.

Immunohistochemistry

Paraffin-embedded tissue sections were cut into standard 4 μm sections, deparaffinised in xylene and rehydrated through graded alcohol solutions. Antigen retrieval was performed for 15 min at 98 °C in citrate buffer (10 mmol/L, pH 6.0) in a microwave oven. Endogenous peroxidases were inactivated by immersing the sections in 0.3% hydrogen peroxide for 20 min. The primary antibody was a rabbit anti-human polyclonal CD133 (dilution 1 : 100; Abcam, Inc., Cambridge, UK) and the sections were incubated overnight at 4 °C in a humidified chamber. Antibodies were detected by EnVision™ + kit (Dako, Carpinteria, CA). Diaminobenzidine was used as the chromagen and then counterstained with haematoxylin. The negative control was performed by incubating samples with phosphate-buffered saline.

### Table 1

| Variable    | n  | Low | High | p-value |
|-------------|----|-----|------|---------|
| Age         |    |     |      | 0.78    |
| < 50        | 28 | 17  | 11   |         |
| > 50        | 35 | 20  | 15   |         |
| Gender      |    |     |      | 0.819   |
| Male        | 50 | 29  | 21   |         |
| Female      | 13 | 8   | 5    |         |
| Tumour size |    |     |      | 0.148   |
| < 5.0 cm    | 18 | 8   | 10   |         |
| > 5.0 cm    | 45 | 29  | 16   |         |
| AFP         |    |     |      | 0.025   |
| < 400 μg/L  | 25 | 19  | 6    |         |
| > 400 μg/L  | 38 | 18  | 20   |         |
| Tumour grade|    |     |      | < 0.001 |
| Well        | 18 | 16  | 2    |         |
| Moderate    | 30 | 18  | 12   |         |
| Poor        | 15 | 3   | 12   |         |
| Tumour stage|    |     |      | 0.007   |
| I–II        | 24 | 20  | 4    |         |
| III–IV      | 39 | 17  | 22   |         |

HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.
Evaluation of CD133 immunostaining
Six normal adult and four fetal liver specimens were used as negative and positive controls. Immunostained slides were independently evaluated by two pathologists, blinded with respect to the clinical features. The quality of staining was evaluated based on the previous studies regarding the expression of CD133 in various tissues (13, 34, 35). Briefly, immunostained cells, excluding endothelial ones, were counted in 4–5 random and non-overlapping fields at high magnification (400×), and were expressed as a percentage of the total number of nuclei counted. For statistical analysis of the increased expression of CD133, patients were divided into two groups using the median value of CD133 expression (1.32%) in all HCC specimens. The expression of CD133 was considered ‘high’, if the percentage was equal to or greater than the median and ‘low’, if less than the median.

Statistical analysis
Statistical analysis and graphical presentation were performed using the spss (v10.0) software for Windows (SPSS Inc., Chicago, IL). The clinicopathological parameters were compared with increased CD133 expression using the Mann–Whitney U-test or the Kruskal–Wallis H-test according to the immunoreactive score of each tissue section. Disease-free and overall survival was calculated using the Kaplan–Meier method, and the resulting curves were compared by the log-rank test. The influence of various clinicopathological features on survival was assessed with the Cox proportional hazards regression model in a stepwise manner. p < 0.05 was considered significant.

Results
CD133 expression in HCC tissues
The expression of CD133 was detected in four fetal livers from the 20th to the 32nd gestational week (Figure 1A). On contrary, all (6/6) normal adult liver cells lacked CD133 immunoreactivity (Figure 1B). CD133-positive tumour cells were found frequently in all 63 HCC cases. These cells were scattered, but occasionally were distributed in groups (Figure 1C–E). CD133 immunoreactivity was present predominantly in the cytoplasm or membrane of cancerous hepatic cells. In addition, immunoreactivity was also found in some stromal cells (Figure 1D). In the HCC tumour samples, 1.32 (±0.81%) of the cells were CD133 positive. Therefore, 26 patients had tumours with high CD133 expression and 37 had tumours with low CD133 expression.

To investigate the clinical role of CD133 during hepatocarcinogenesis, CD133 expression was compared with clinicopathological features. Increased CD133 expression in HCC patients was associated with three tumour-related variables (Table 1). Increased CD133 expression was more frequent in tumours with stages III or IV than in tumours with stages I or II. Increased CD133 expression also correlated with higher histological grade and elevated serum AFP levels. However, there was no difference between the expression of CD133 and other clinicopathological features in HCC including patient age, sex and tumour size.

Disease-free survival and HCC expressing CD133
Most HCC patients die because of disease recurrence after hepatectomy. Therefore, we evaluated the association between increased CD133 expression and disease-free survival. The 5-year disease-free survival rates were 38.24% in patients with low CD133 expression, but only 7.69% in patients with high CD133 expression. Kaplan–Meier analysis showed that patients with increased CD133 expression had a significantly shorter disease-free survival time than those with low expression (Figure 2). Univariate Cox regression analysis also identified that both tumour stage and CD133 expression were significantly associated with disease-free survival (Table 2). Variables that were significant in univariate analysis were entered into multivariate analysis, and only increased CD133 expression correlated with disease-free survival in HCC (Table 3). Patients with increased CD133 staining had a worse postoperative disease-free survival than those without demonstrating the prognostic value of CD133 staining of tumour tissues.

Overall survival and HCC expressing CD133
To investigate the usefulness of CD133 expression in determining the postoperative prognosis of patients with HCC, the association between increased CD133 expression and overall survival was evaluated using Kaplan–Meier survival curves with the log-rank test and confirmed with Cox regression models. The 5-year overall survival rates were 50% and 19.23% in patients with low and high CD133 expression respectively. Patients with increased CD133 expression had a significantly shorter overall survival than those with low expression (Figure 3). Univariate Cox regression analysis also identified that clinical variables including tumour stage, AFP levels and CD133 expression were significantly associated with overall survival (Table 2). Multivariate Cox regression analysis revealed that increased CD133 expression and
tumour stage were significant predictors of overall survival in HCC (Table 3). These results demonstrate that increased CD133 staining correlates with worse long-term survival for patients.

Discussion

CD133-positive stem cells may play an essential role in carcinogenesis including HCC. In this study, we demonstrated that CD133, a stem cell marker, was expressed in fetal livers but not in normal adult livers and was re-expressed in cancerous livers. This pattern of expression is similar to that of AFP (21), as oncodevelopmental marker, suggesting that CD133 may play an oncogenic role in HCC. We also found that increased expression of CD133 correlated with higher tumour stage and histological grade. This result is consistent with previous reports, that
increased CD133 expression is associated more frequently with higher pathological stage in other malignancies (13,29,36).

Previous studies demonstrated that expression of CD133 was restricted to stem cells (23,24,37). Interestingly, elevated serum AFP level, another stem cell marker in HCC, was associated with increased CD133 expression, confirming the stem cell phenotype of CD133-positive cells. Accordingly, our findings suggest that CD133-positive cells may be involved in the development of HCC. In contrast, other studies showed no significant correlation between CD133 expression and the clinicopathological features of HCC (35). The discrepancies may result from the use of different antibodies, protocols and scoring systems in these studies, or it may reflect the heterogeneity of HCC. We found that increased CD133 expression correlates with disease-free and overall survival. Further analysis revealed that increased CD133 expression is an independent factor of poor prognosis in both disease-free and overall survival.

Table 2 Univariate analysis of disease-free or overall survival in 60 patients with HCC

| Variables          | n   | p (disease-free survival) | p (overall survival) |
|--------------------|-----|---------------------------|----------------------|
| Age                |     |                           |                      |
| £50                | 28  | 0.673                     | 0.197                |
| >50                | 35  |                           |                      |
| Gender             |     |                           |                      |
| Male               | 50  | 0.978                     | 0.365                |
| Female             | 13  |                           |                      |
| Tumour size        |     |                           |                      |
| £5.0 cm            | 18  | 0.105                     | 0.152                |
| >5.0 cm            | 45  |                           |                      |
| AFP                |     |                           |                      |
| £400 µg/l          | 25  | 0.102                     | 0.036                |
| >400 µg/l          | 38  |                           |                      |
| Tumour grade       |     |                           |                      |
| Advanced           | 18  | 0.132                     | 0.217                |
| Moderate           | 30  |                           |                      |
| Poor               | 15  |                           |                      |
| Tumour stage       |     |                           |                      |
| I–II               | 24  | 0.009                     | 0.005                |
| III–IV             | 39  |                           |                      |
| CD133              |     |                           |                      |
| Low                | 34  | <0.001                    | 0.001                |
| High               | 26  |                           |                      |

HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.

Table 3 Multivariate analysis of disease-free or overall survival in 60 patients with HCC

| Variables          | Relative risk (95% CI) | p-value |
|--------------------|------------------------|---------|
| Disease-free survival |                         |         |
| CD133              | 2.445 (1.230–4.859)    | 0.011   |
| Overall survival   |                         |         |
| CD133              | 2.443 (1.173–5.088)    | 0.017   |
| Tumour stage       | 2.793 (1.246–6.261)    | 0.013   |

HCC, hepatocellular carcinoma; CI, confidence interval.
A series of genetic and epigenetic alterations in mature hepatocytes contribute to multistep hepatocarcinogenesis (38–40). However, according to the CSC hypothesis, a rare fraction of CSCs contributes to tumour initiation, progression, recurrence and metastasis. Moreover, many prior studies revealed CSC properties in various human tumours, which could account for the malignancy of these tumours such as recurrence, metastasis and drug resistance (1,8). In particular, recent studies confirmed that CD133-positive cells have stem cell properties and are responsible for multidrug resistance in cell lines used as a HCC model (41) supporting the CSC hypothesis. However, little is known about the relationship between CSCs and the clinical symptoms of HCC. This is the first study to show that increased CD133 expression, a stem cell marker, is an independent prognostic factor in patients with HCC. The amount of CSCs may impact malignant characteristics in HCC such as tumour stage, histological grade and AFP level, and therefore be relevant to patients’ prognosis. Further studies are needed to isolate and identify the stem cell properties of CD133-positive cells in HCC specimens.

In conclusion, this study demonstrates that reactivated CD133-positive cells are present frequently in HCC. Additionally, increased CD133 expression correlates with higher pathological stage in HCC and thus indicates a poor prognosis for patients with HCC, supporting the CSC hypothesis.

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Author contributions

WJ Song designed the study, performed and evaluated the immunohistochemistry and wrote the manuscript. HM Li and KS Tao evaluated the clinical records, did the statistical analysis and revised the manuscript. R Li, ZS Song, QC. Zhao and FQ Zhang participated in the design of the study and assisted in the method development. KF Dou designed and led the study, did the evaluation of immunohistochemistry, and wrote the manuscript. All authors have read and approved the final manuscript.

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