The Expression Status and Prognostic Value of Cancer Stem Cell Biomarker CD133 in Cutaneous Squamous Cell Carcinoma

Rui Xu, BS; Mu-Yan Cai, MD; Rong-Zhen Luo, MD; Xin Tian, MD; Jian-de Han, MD; Mu-Kai Chen, MD

IMPORTANCE The CD133 protein has been considered a key biomarker of cancer stem cells in various cancers. However, the expression status and prognostic significance of CD133 in cutaneous squamous cell carcinoma (cSCC) are poorly understood.

OBJECTIVE To investigate the expression of cancer stem cell biomarker CD133 in cSCC tissue and its effect on clinicopathological features and outcomes in patients with cSCC.

DESIGN, SETTING, AND PARTICIPANTS Immunohistochemistry was performed on a tissue microarray to investigate the expression levels of CD133 in cSCC tissue. Receiver operating characteristic curve analysis, Kaplan-Meier plots, and a Cox proportional hazards regression model were applied to analyze the data. Samples were obtained from the archives of the First Affiliated Hospital, Sun Yat-Sen University Cancer Center, and Guangzhou Institute of Dermatology and Venerology. In total, 165 paraffin-embedded clinicopathological samples from 165 patients were obtained from the archives of hospitals between June 1, 1996, and December 31, 2010. Follow-up data were available for these cases.

MAIN OUTCOMES AND MEASURES The CD133 expression in cSCC tissue, correlation of CD133 expression with clinicopathological features of cSCC, and association of CD133 expression with prognosis in patients with cSCC.

RESULTS Based on the receiver operating characteristic curves, the cutoff value for high CD133 expression was defined as greater than 65% of tumor cells positively stained. High CD133 expression was observed in 50.9% (84 of 165) of the cSCC samples and in 16.7% (5 of 30) of adjacent nonmalignant epithelial tissue samples (P = .001). High CD133 expression was positively correlated with poorly differentiated cSCC (48.0% [73 of 150] for well to moderately differentiated vs 84.6% [11 of 30] for poorly differentiated, P = .01) and with advanced tumor stage (45.5% [55 of 121] for stage I-II vs 65.9% [29 of 44] for stage III, P = .02). In univariable survival analysis, high CD133 expression was correlated with poor prognosis (mean survival, 63.4 vs 95.7 months; P < .001). In multivariable analysis, CD133 expression was an independent prognostic factor for cSCC (hazard ratio, 1.9152; 95% CI, 1.1950-3.3495; P = .02).

CONCLUSIONS AND RELEVANCE High CD133 expression is associated with poorly differentiated and advanced-stage cSCC. High CD133 expression was also correlated with poor prognosis in patients with cSCC. It may serve as a useful biomarker to predict prognosis in patients with cSCC.
Cutaneous squamous cell carcinoma (cSCC) is the second most common form of nonmelanoma skin cancer after basal cell carcinoma and accounts for 20% of cutaneous malignant neoplasms and most nonmelanoma skin cancer deaths. An estimated 700,000 new cases of cSCC are diagnosed annually in the United States. Similar incidence trends have been noted in China, and because of the growing elderly Chinese population in recent decades, cSCC will be an emerging health burden.

Most cSCCs occur on visible areas of the skin such as the head, face, and neck, which may be treated with conservative measures, including surgery, irradiation, and chemotherapy. However, 8% of invasive cSCCs recur, and 5% metastasize within 5 years. Therefore, recent cSCC studies have focused on the discovery of specific molecular biomarkers that could serve as reliable prognostic indicators, but novel biomarkers to predict outcomes in patients with cSCC remain limited.

Cancer stem cells (CSCs) are a small population of tumor cells and are the only cells that possess the ability to initiate and maintain tumor growth. Recently, CSCs have been identified and isolated from several human solid cancers and were suggested to be a source of cancer cells responsible for the initial disease, progression, metastasis, recurrence, and resistance to therapy. There are various stem cell biomarkers of different cellular origins for CSCs. The CD133 protein, also known as human prominin 1, is a transmembrane glycoprotein that has been considered a putative and important CSC biomarker in various tumors, including melanoma and lung, breast, colon, and brain cancer. Moreover, high CD133 expression was correlated with poor clinical outcomes in some cancers.

A few studies have investigated CD133 expression in skin cancers, including melanoma, but, to our knowledge, there has been little consideration of CD133 expression in cSCC tissue. In this study, a tissue microarray was used to examine the distribution and frequency of CD133 expression in cSCC tissue and in adjacent nonmalignant epithelial tissue by immunohistochemistry (IHC). Receiver operating characteristic (ROC) curve analysis was applied to define a cutoff value for high CD133 expression. In addition, clinicopathological and prognostic significance of CD133 expression in cSCC tissue was analyzed.

Methods

Patients and Tissue Samples

For this study, paraffin-embedded clinicopathological samples were obtained from the archives of the First Affiliated Hospital, Sun Yat-Sen University Cancer Center, and Guangzhou Institute of Dermatology and Venerology, between June 1, 1996, and December 31, 2010. In total, 165 patients with cutaneous SCC were included in the study who met the following criteria: (1) the diagnosis was confirmed by a dermatopathologist (M.-Y.C. or R.-Z.L.), (2) they had a primary tumor without any preoperative anticancer treatment, and (3) a resected tissue specimen and follow-up data were available. These cSCCs occurred in areas of sun exposure, including the head, face, and neck. Included were 111 men (67.3%) and 54 women (32.7%), with a mean age of 54.7 years. The mean follow-up time was 51.1 months (median, 50.6 months; range, 2.8-110.0 months).

Patients whose cause of death was unknown or who had evidence of immunosuppression or arsenic exposure were excluded from the study. Clinicopathological characteristics of the study patients, including age, sex, pT and pN classification, and tumor location, size, grade, and stage are listed in Table 1. The TNM classification of tumors was based on the World Health Organization tumor classification criteria. The TNM classification of cutaneous carcinoma was defined according to the Union for International Cancer Control. The Institute Research Medical Ethics Committee of Sun Yat-Sen University approved the study. All patient data were deidentified.

Tissue Microarray Construction

Tissue microarrays were constructed according to a method described previously. In brief, formalin-fixed, paraffin-embedded tissue blocks were placed onto glass slides and stained with hematoxylin-eosin, and tumor areas were verified and marked by a senior pathologist (M.-Y.C.). Triplicate cylindrical tissue samples 0.6 mm in diameter were obtained from the tissue blocks that included the most representative area from 165 patients. These samples were re-embedded into paraffin blocks at defined positions (ie, cylindrical holes) with a tissue array Beecher instrument and used for immunohistochemical staining of CD133.

Immunohistochemistry

Tissue microarray slides were dried overnight at 37°C, deparaffinized in xylene, rehydrated through graded alcohol, immersed in 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity, and antigen retrieved by pressure cooking for 3 minutes in EDTA buffer (pH 8.0). The slides were then preincubated with 10% normal goat serum at room temperature for 30 minutes to reduce nonspecific reactions. Next, the slides were incubated with mouse monoclonal anti-CD133 human prominin 1 (EMD Millipore) at a concentration of 3 ng/mL for 2 hours at room temperature. Then, the slides were incubated with a secondary antibody (Envision; Dako) for 1 hour at room temperature and stained with 3,3′-diaminobenzidine. Finally, the sections were counterstained with Mayer hematoxylin, dehydrated, and mounted. A negative control was obtained by replacing the primary antibody with normal mouse IgG. Known immunostaining-positive slides (of hepatocellular carcinoma tissue) were used as positive controls.

IHC Evaluation

Immunohistochemistry was performed as described previously. The CD133 expression was examined by 2 independent pathologists (M.-Y.C. and R.-Z.L.) who were masked to clinicopathological patient data. A semiquantitative immunoreactive method was used to measure CD133 by counting the number of positive tumor cells among the total number of tumor cells per high-power field. The denominator was ob-
tained by calculating the percentage of positive vs negative cells within the bulk of the cellular part of the tumor. Values were assigned using 5% increments (range, 0%-100%).

Selection of the Cutoff Value for High CD133 Expression

A ROC curve analysis was used to establish a cutoff value for high CD133 expression in tumors using the shortest distance from the curve to the point with the maximum sensitivity and specificity (0,0, 1,0). For CD133, the sensitivity and specificity were plotted for each outcome under examination, generating various ROC curves (Figure 1). Tumors at or below the cutoff value were designated as having low CD133 expression, and tumors above the cutoff value were designated as having high CD133 expression. Using ROC curve analysis, we dichotomized the following clinicopathological data: tumor size (≤5 or > 5 cm), tumor grade (well to moderately or poorly differentiated), pT classification (T1-T2 or T3-T4), pN classification (N0 or N1), tumor stage (I-II or III-IV), and survival status (death from cSCC or censored).

Statistical Analysis

Statistical analyses were performed using a software package (SPSS, version 13.0; IBM). An ROC curve analysis was used to determine the cutoff value for high CD133 expression. Correlation of CD133 expression with clinicopathological features of patients with cSCC was evaluated by the χ2 test. Univariate and multivariable survival analyses were performed using a Cox proportional hazards regression model. Survival curves were plotted by the Kaplan-Meier method. Two-tailed P < .05 was considered statistically significant.

Results

CD133 Expression in cSCC Tissue and Adjacent Nonmalignant Epithelial Tissue

The CD133 expression was evaluated in 165 cSCC samples, and immunoreactivity of CD133 in cSCC tissue ranged from 0% to 100%. The CD133 IHC staining in cSCC tissue and adjacent nonmalignant epithelial tissue showed mixed membranous and cytoplasmic staining in tumor cells (Figure 2). A ROC curve analysis defined CD133 expression as high when the CD133 expression percentage was above 65%. Values at or below this cutoff were considered low expression. In this study, 81 of 165 cSCC samples (49.1%) had low CD133 expression. High CD133 expression was demonstrated in 84 of 165 cSCC samples (50.9%) and in 5 of 30 adjacent nonmalignant epithelial tissue samples (16.7%) (P = .001, Fisher exact test).

Selection of a Cutoff Value for High CD133 Expression

The ROC curves for each clinicopathological parameter (Figure 1) show the point on the curve closest to the point that
maximizes the sensitivity and specificity (0.0, 1.0) for the outcome as described previously. The cutoff value for CD133 high expression was defined as greater than 65% of tumor cells with positive CD133 staining. High CD133 expression was associated with poorly differentiated and advanced-stage cSCC and with poor survival (Figure 1).

**Association of CD133 Expression With Clinicopathological Features of cSCC**

High CD133 expression was greater in patients with poorly differentiated cSCC ($P = .01$) and with advanced tumor stage ($P = .02$) (Table 1). There was no significant correlation between CD133 expression and the other clinicopathological parameters, including patient age, sex, tumor location and size, and $pT$ and $pN$ classification ($P > .05$ for all).

**Association of Clinicopathological Features With CD133 Expression and Survival of Patients With cSCC in the Univariable Survival Analysis**

To confirm the representativeness of the cSCC samples in our study, the sample was analyzed according to established prognostic factors for patient survival. The Kaplan–Meier analysis demonstrated a significant effect of several well-known clinicopathological prognostic factors, including tumor size ($P = .03$), $pN$ classification ($P = .009$), and tumor stage ($P = .003$), on overall patient survival (Table 2). Moreover, an assessment of recurrence-free survival (Figure 3A) and overall survival (Figure 3B) ($P < .001$ for both) showed that high CD133 expression was correlated with poor survival in patients with cSCC.

**Independent Prognostic Factors for cSCC in the Multivariable Cox Proportional Hazards Regression Model**

Several features of cSCC observed to be prognostic factors by univariable analysis, including CD133 expression and clinicopathological variables that were significant by univariable analysis (ie, tumor size, $pN$ classification, and tumor stage), were further evaluated by multivariable analysis. A multivariable regression analysis based on the Cox proportional hazards regression model was applied to test the independent value of each parameter in predicting overall survival. The results showed that high CD133 expression was an independent prognostic factor for poor overall survival (hazard ratio, 1.915; 95% CI, 1.195-3.349; $P = .02$) (Table 2). Tumor stage was found to be another independent prognostic factor for poor overall survival (hazard ratio, 1.812; 95% CI, 1.012-3.246) ($P = .045$).
Discussion

A key biomarker of CSCs in various cancers, CD133 was originally identified as a transmembrane glycoprotein in normal hematopoietic stem and progenitor cells that were involved in proliferation, self-renewal, and multilineage differentiation. In the present study, high CD133 expression was observed in many cSCC samples, and it was more frequently observed in cSCC tissue than in adjacent nonmalignant epithelial tissue. High CD133 expression was positively correlated with poorly differentiated cSCC and with advanced tumor stage. In addition, high CD133 expression and other clinicopathological prognostic factors (eg, tumor stage) were strong and independent predictors of poor overall survival by univariable and multivariable analyses.

Previous evidence has suggested that a CD133-positive subpopulation of multipotent cells has biological features of CSCs and can be used to identify putative CSCs of solid tumors. High CD133 expression levels were associated with poor prognosis in solid tumors, including lung, breast, colon, and brain cancers. Recent data document CD133 expression in skin tumors, including staining patterns in melanoma and cSCC. Our results showed that high CD133 expression was observed in many of our cSCC samples and was associated with poorly differentiated tumor and with advanced tumor stage. It was previously reported that tumor-initiating cells are present in human cSCC, are enriched in the CD133-positive cSCC subpopulation, and exhibit the stem cell property of self-renewal. These findings provided evidence that CSCs or CD133 upregulation may have an important role in the tumorigenic process and development of cSCC. Keratinocytes with dysfunctional p53 as a result of irradiation cannot undergo apoptosis but demonstrate clonal expansion, which is manifested clinically as the development of actinic keratosis and SCC in situ. Although the mechanism of CD133 and CSCs in the tumorigenesis and progression of cSCC has not been identified, studies have indicated that the CD133-positive cell population demonstrates significant resistance to transforming growth factor β–induced apoptosis and tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis compared with CD133-negative cells. Moreover, silencing of CD133 can impair the self-renewal and tumorigenic capacity of tumor cells. The results of these studies suggested that uncontrolled proliferation of CSCs (CD133 cells) may lead to SCC in situ and invasive cSCC. Furthermore, the findings herein suggested that CD133 expression in cSCC tissue may facilitate an increase in malignant clinical features or lead to a worse prognosis. Therefore, IHC investigation of CD133 expression may be a good tool to identify patients with higher risk of worse cSCC progression. New treatment strategies for patients with poorly differentiated cSCC may be developed that target tumors containing a higher frequency of CD133 (a CSC biomarker).

A few limitations are inherent to the present study. First, our study was retrospective and included a single biomarker (CD133). Second, the study enrolled few patients and lacked standardization with respect to the follow-up period. Third, although correlation of CD133 expression in SCC tissue with patient survival was the focus of our study, the underlying mechanism by which CD133 affects prognosis in patients with cSCC remains elusive and will require future investigation.

Figure 2. CD133 Expression in Cutaneous Squamous Cell Carcinoma Tissue and Adjacent Nonmalignant Epithelial Tissue by Immunohistochemistry

The top panels show original magnification ×100. A, High CD133 expression is observed in a case in which more than 90% of tumor cells revealed positive immunostaining of CD133 in their membrane and cytoplasm. B, A second case demonstrates low CD133 expression in which less than 30% of tumor cells revealed positive immunostaining of CD133 in their membrane and cytoplasm. C, Almost no CD133 expression is shown in a third case. D, Adjacent nonmalignant epithelial tissue shows almost no CD133 expression. The bottom panels show original magnification ×400 of the insets.
Table 2. Univariable and Multivariable Analyses of Prognostic Factors in 165 Patients With Primary Cutaneous Squamous Cell Carcinoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of Patients</th>
<th>Univariable Analysisa</th>
<th>Multivariable Analysisb</th>
<th>P Value</th>
<th>HR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤54.7°</td>
<td>87</td>
<td>84.5</td>
<td>.14</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;4.7</td>
<td>78</td>
<td>72.6</td>
<td>.20</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>54</td>
<td>80.0</td>
<td>.70</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>111</td>
<td>78.8</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>75</td>
<td>73.1</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Face</td>
<td>51</td>
<td>78.5</td>
<td>.20</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Neck</td>
<td>39</td>
<td>88.3</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor size, cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>76</td>
<td>85.8</td>
<td>.03</td>
<td>1 [Reference]</td>
<td>1.464 (0.819-2.617)</td>
<td>.20</td>
</tr>
<tr>
<td>&gt;5</td>
<td>89</td>
<td>72.9</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well to moderately differentiated</td>
<td>152</td>
<td>81.1</td>
<td>.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>13</td>
<td>56.3</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>pT Classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>76</td>
<td>85.0</td>
<td>.07</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>T3-T4</td>
<td>89</td>
<td>74.0</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>pN Classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>134</td>
<td>83.6</td>
<td>.009</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>N1</td>
<td>31</td>
<td>58.3</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>121</td>
<td>85.4</td>
<td>.003</td>
<td>1 [Reference]</td>
<td>1.812 (1.012-3.246)</td>
<td>.045</td>
</tr>
<tr>
<td>III-IV</td>
<td>44</td>
<td>60.2</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD133 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>81</td>
<td>95.7</td>
<td>&lt;.001</td>
<td>1 [Reference]</td>
<td>1.915 (1.095-3.349)</td>
<td>.02</td>
</tr>
<tr>
<td>High</td>
<td>84</td>
<td>63.4</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; NS, not significant.

Table 3. Kaplan-Meier Survival Analysis of CD133 Expression in Patients With Cutaneous Squamous Cell Carcinoma

A. Total probability of recurrence-free survival is shown. B. Total probability of overall survival is shown.

The numbers at risk are 81 with low expression and 84 with high expression.
Conclusions

High CD133 expression was correlated with poorly differentiated cSCC and with advanced tumor stage. Moreover, high CD133 expression was an independent prognostic factor for an unfavorable prognosis in patients with cSCC. These findings suggest that CD133 expression as examined by IHC may be used as a biomarker of shortened survival in patients with cSCC and could be a promising molecular therapeutic target for cSCC.

REFERENCES