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Over-Expression of *CD200* Predicts Poor Prognosis in Cutaneous Squamous Cell Carcinoma

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: *CD200* is reported to be involved in tumor progression and can serve as a prognostic marker in several cancers. The purpose of this study was to evaluate the prognostic significance of *CD200* in cutaneous squamous cell carcinoma (CSCC).

Material/Methods: The relative mRNA and protein expression of *CD200* in the tumor tissues and corresponding normal tissues of 102 CSCC patients were detected by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis, respectively. The chi-square test was used to analyze the association between *CD200* expression and clinical features of CSCC patients. In addition, the overall survival of the patients according to the expression level of *CD200* was estimated by Kaplan-Meier analysis and the prognostic significance of the gene was analyzed by Cox regression analysis.

Results: Increased expression of *CD200* was detected in the tumor tissues compared with the corresponding normal tissues both at mRNA and protein level. And *CD200* expression level was associated with tumor differentiation grade (P=0.041) and clinical stage (P=0.004). Patients with high expression level of *CD200* had a shorter overall survival than those with low expression (31.3 months *vs.* 41.9 months) and there was a significant difference between them (log-rank test, P<0.001). Cox regression analysis indicated that *CD200* could be an independent marker for the prognosis of CSCC.

Conclusions: *CD200* is up-regulated and may be a novel biomarker for the prognosis in CSCC, and it may be a potential therapeutic target for CSCC.

MeSH Keywords: **Antigens, CD • Prognosis • Skin Neoplasms**

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Background

Cutaneous squamous cell carcinoma (CSCC) is one of the most common skin cancers in the world, accounting for approximately 20% of all cutaneous malignancies [1]. The cancer often occurs on the highly visible areas of the skin, such as the head, neck, and face [2]. CSCC in most patients can be completely eradicated with surgery or other dermatological treatments, but some cases suffer with a substantial risk of metastasis and high mortality [3–5]. Therefore, a number of studies have concentrated on exploring of the molecular markers, which would provide responsibly prognostic information for tumor treatments. However, the specific biomarkers for CSCC prognosis, which could provide significantly clinical information, remain substantially limited [2].

CD200, also known as *OX-2*, is a member of the Ig superfamily (IgSF) of proteins and is important in the regulation and fine control of immune responses, as well as in helping to maintain immune homeostasis [6,7]. It is a membrane glycoprotein and plays a role in inducing signaling in cells expressing its cognate cell surface receptor, the *CD200* receptor (CD200R) [7]. In *CD200*-deficient mouse models, hyper-activation of macrophages was observed and the immune response to autoimmune disease was also enhanced [8]. Recently, the abnormal expression of *CD200* has been detected in several types of cancer, such as plasma cell myeloma, head and neck squamous cell carcinoma, and melanoma cells [9–11]. Daniel et al. reported that the expression level of *CD200* in tumor tissues was higher than that in normal tissues in CSCC patients and that the gene might accelerate tumor progression [12]. However, the clinical significance of *CD200* in CSCC has rarely been reported.

In this study we aimed to investigate the expression level of *CD200* in clinical CSCC specimens and normal tissues. Moreover, the relationship between *CD200* expression and clinical factors of patients with CSCC was analyzed. The clinical significance of *CD200* in CSCC patients was estimated via Kaplan-Meier and Cox regression analysis.

Material and Methods

Patients and tissue specimens

We collected 102 CSCC patients who were confirmed by pathological and clinical diagnoses in General Hospital of Beijing Military Region from October 2009 to February 2015. The study was approved by the Ethics Committee of the hospital. None of the patients had received any chemotherapy or radiotherapy before the surgery. Written informed consents were signed by all participants in advance.

Tumor tissues and corresponding normal tissues of the patients were obtained and frozen in liquid nitrogen immediately, then stored at -80°C until use. The clinicopathologic characteristics of patients with CSCC, including age, sex, tumor size, tumor differentiation grade, and clinical stage, were recorded in a database. All of the patients were arranged for 5-year follow-up and the information about the clinical features and survival were collected for analyzing the clinical significance of *CD200*.

Total RNA extraction and qRT-PCR analysis

Total RNA was extracted from all the specimens by Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNAse was used for the residuary DNA in the extracted RNA. The concentration of the RNA was detected by UV absorbance (A260/A280), and 1% agarose gel electrophoresis was used for evaluating its quality. Reverse transcription was conducted to synthesize the first chain of cDNA using Prime Scrip RT reagent kit (Takara, Dalian, China). Then RT-PCR reaction was performed with SYBR Green assay (Takara, Dalian, China). *GAPDH* acted as internal control and $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression of *CD200* data analyses. The sequences of the primers used in this study are listed in Table 1.

Western blot analysis

Total protein was extracted from all samples and separated by 10% SDS-PAGE gels. Then the brands were transferred to PVDF membrane (Bio-Rad, CA, USA) after blocking for 1 h with 5% non-fat milk. Subsequently, the membranes were

Table 1. The primer sequences of *CD200* and *GAPDH* in RT-PCR reaction.

Genes		Sequences
CD200	Forward	GAGCTCCAGGCGCACATCCGC
	Reverse	TGCGGATGTGCGCCTGGAGCTC
GAPDH	Forward	GAAGGTGAAGCTCGCAGTC
	Reverse	GAAGATGGTGATGGGATTTC

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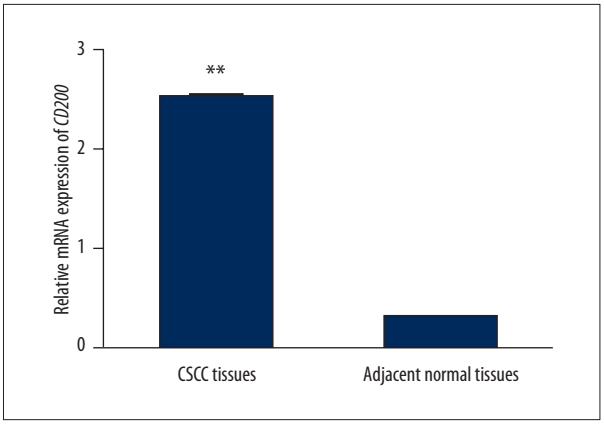

Figure 1. Relative mRNA expression of *CD200* in tumor tissues and corresponding normal tissues of CSCC patients (*GAPDH* as normalized control). *CD200* expression was higher in tumor tissues than in adjacent normal tissues ($P<0.001$).

incubated with primary monoclonal antibodies against *CD200* or GAPDH (at a 1:1000 dilution) for 1 h and washed 6 times over a period of 1 h in TBS/Tween-20. Blots were then incubated at 1:10000 with anti-goat, 500 µg/0.5ml IgG HRP (Jackson ImmunoResearch) in blocking buffer for 1 h and washed 6 times over a period of 1 h in TBS/Tween-20. Finally, the membranes were developed using ECL and exposed to X-ray film for 30 s.

Statistical analysis

All data are showed as mean ± standard deviation (SD). The difference between the 2 groups was analyzed by Students' t test. Association between *CD200* expression and the clinical features were estimated by chi-square test. Kaplan-Meier analysis was used for evaluating the overall survival of the patients according to the *CD200* expression. The prognostic significance of the gene was analyzed by Cox regression analysis. All of the statistical analyses were performed in SPSS 18.0 software (SPSS Inc, IL, USA), and Sigma Plot software (Systat Software Inc., CA, USA) was used for drawing. $P<0.05$ was considered to be statistically significant.

Results

The expression level of *CD200* was increased in tumor tissues

Relative mRNA and protein expression of *CD200* was detected by qRT-PCR and Western blot analysis, respectively. As shown in Figure 1, relative mRNA expression of *CD200* was significantly higher in tumor tissues than in adjacent normal tissues ($P<0.001$). The protein expression of *CD200* was also increased in tumor tissues compared to adjacent normal tissues ($P<0.001$, Figure 2)

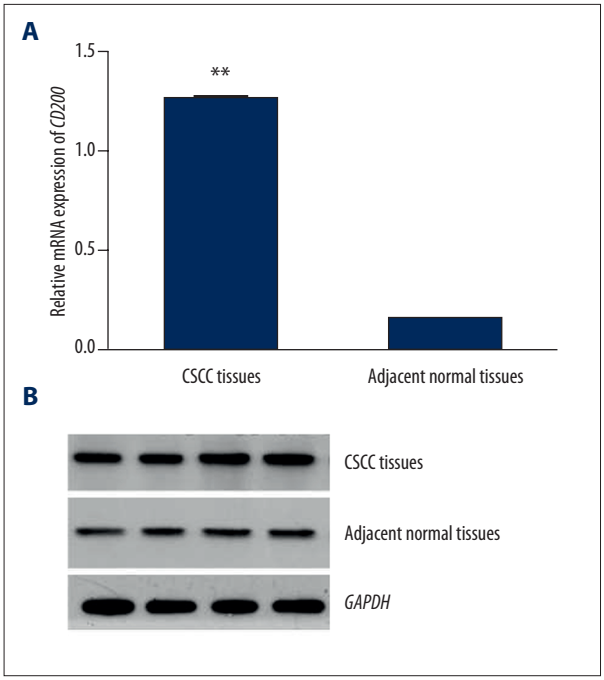

Figure 2. Relative protein expression of *CD200* in tumor tissues and corresponding normal tissues of CSCC patients. *CD200* protein expression was higher in tumor tissues than in adjacent normal tissues ($P<0.001$).

Correlation between *CD200* expression and clinicopathologic characteristics of CSCC patients

All clinical data are listed in Table 2. The 102 CSCC patients included 39 males and 63 females, with average age of 57.4 years. The patients were divided into a high expression group and a low expression group according to the median expression level of *CD200*. The results indicated that *CD200* expression was significantly associated with tumor grade of differentiation ($P=0.041$) and clinical stage ($P=0.004$). However, no distinct differences between sex, age, and *CD200* expression were observed (Table 2).

Association between *CD200* expression and overall survival of patients with CSCC

To explore the prognostic value of *CD200*, we conducted 5-year follow-up with all patients. According to Kaplan-Meier analysis, patients with high expression level of *CD200* had a shorter overall survival than those with low expression (31.3 months *vs.* 41.9 months, log-rank test, $P<0.001$, Figure 3). Then a univariate and multivariate analysis were performed to estimate the prognostic value of *CD200* using Cox regression analysis. The result demonstrated that high expression of *CD200* (HR=4.558, 95%CI=2.397–8.666, $P=0.000$), tumor differentiation grade (HR=1.833, 95%CI=1.036–3.242, $P=0.037$), and clinical stage (HR=2.374, 95%CI=1.369–4.118, $P=0.002$) were

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Table 2. Association between *CD200* expression and the clinical features of CSCC patients.

Characteristics	Cases (n)	*CD200* expression		χ^2	*P*
		High (n, %)	Low (n, %)		
Gender				1.533	0.216
Male	39	18 (46.2%)	21 (53.8%)		
Female	63	37 (58.7%)	26 (41.3%)		
Age				0.532	0.466
≥55	59	30 (50.8%)	29 (49.2%)		
<55	43	25 (58.1%)	18 (41.9%)		
Tumor size				0.710	0.399
≥5 cm	54	37 (68.5%)	17 (31.5%)		
<5 cm	48	18 (37.5%)	30 (62.5%)		
Tumor differentiation grade				4.156	0.041
Well to moderate	67	41 (61.2%)	26 (38.8%)		
Poor	35	14 (40.0%)	21 (60.0%)		
Clinical stage				8.447	0.004
I+II	45	17 (37.8%)	28 (62.2%)		
III+IV	57	38 (66.7%)	19 (33.3%)		

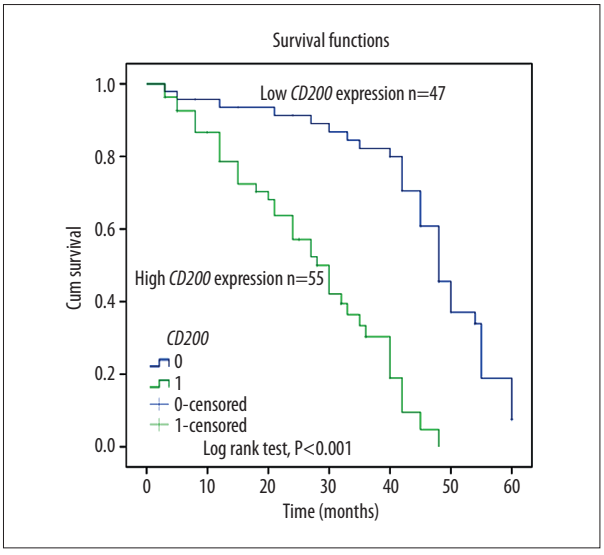

Figure 3. Overall survival of CSCC patients according to the expression of *CD200*. Patients with high level of *CD200* had a shorter overall survival time than those with low-expression. There was a significant difference between the groups (log-rank test, *P*<0.001).

tightly related to the prognosis of CSCC and they could act as independent prognostic biomarkers in CSCC.

Discussion

To improve the clinical outcomes of CSCC patients, a growing number of studies have focused on looking for the molecular markers associated with cancer prognosis. *LRIG-1* was proved to be an excellent prognostic indicator in CSCC [13]. *P300*, a member of the histone acetyltransferase family of transcriptional coactivators, was reported to be an independent biomarker in the prognosis of CSCC by Chen et al. [2]. Some members of the HER family might be potential therapeutic and prognostic markers in CSCC due to its association with tumor progression and lymph node metastasis [14]. In this study, we engaged in discovering more molecular markers to better predict the prognosis of patients with CSCC.

CD200 is a cell surface glycoprotein that can inhibit alloimmune and autoimmune responses via its receptor CD200R [15–17]. The immune suppression capacity of *CD200* could maintain homeostasis in various tissues, such as lymphoid cells, the nervous system, and skin [16,18,19]. It might also help protect migratory neoplastic cells [15]. In previous studies, *CD200* was reported to take part in several cancers. For instance, *CD200* was overexpressed and correlated with progression of metastatic melanoma as well as acting as a potential therapeutic target [20]. Siva et al. reported that *CD200* was expressed in ovarian cancer, melanoma, neuroblastoma, and renal carcinoma, thereby

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Table 3. The multivariate analysis adjusted for clinical factors for estimating the prognostic value of *CD200* in CSCC.

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P*	HR	95%CI	*P*
CD200 (high *vs.* low)	5.512	3.035–10.011	0.000	4.558	2.397–8.666	0.000
Gender (male *vs.* female)	1.474	0908–2.395	0.117	–	–	–
Age (≥55 *vs.* <55)	0.932	0.579–1.500	0.773	–	–	–
Tumor size (>5 cm *vs.* <5 cm)	1.312	0.822–2.094	0.256	–	–	–
Tumor differentiation grade (well *vs.* poor)	2.441	1.456–4.091	0.001	1.833	1.036–3.242	0.037
Clinical stage (I+II *vs.* III+IV)	3.239	1.962–5.347	0.000	2.374	1.369–4.118	0.002

potentially suppressing anti-tumor immune responses, but it was absent in prostate, lung, breast, astrocytoma, or glioblastoma [21]. *CD200* co-expression with stem cell markers was found in prostate, breast, brain, and colon cancers [22]. According to Podnos et al., *CD200* expression could prevent the delivery of an immunosuppressive signal and influence metastatic growth, suggesting it as a potential therapy for breast cancer [23]. Zhang et al. detected the expression of *CD200* in acute myeloid leukemia and proved that its antigen expression was related to the poor prognosis of this disease [24]. The over-expression of *CD200* is linked with expansion of suppressive Treg cells and elevation of cytokines, and plays a vital role in the progression of multiple myeloma [25]. The diagnostic and prognostic value of *CD200* was also confirmed in plasma cell myeloma [26]. *CD200* protein was verified to be a prognostic indictor for the poor prognosis of multiple myeloma [24]. Using gene expression data from the databases, Jerome et al. concluded that *CD200* expression is associated with the progression of various cancers, such as bladder cancer, lung cancer, chronic myelogenous leukemia, breast cancer, primary melanoma, metastatic melanoma, prostatic intraepithelial neoplasia, and prostate carcinoma [28]. However, the clinical significance of *CD200* in CSCC is still unclear.

In the present study, we detected the expression of *CD200* both at mRNA and protein level in CSCC tissues and adjacent normal tissues. The over-expression of *CD200* at 2 levels was found, which indicated *CD200* might be an oncogene in CSCC. Based on this result, we explored wether it was involved in the progression of CSCC. Finally, tumor differentiation grade and clinical stage were verified to tightly influence the expression of *CD200*. All of these results suggest that *CD200* is associated with tumor progression in CSCC.

Because the prognostic value of *CD200* was also verified in many malignancies, we investigated the prognostic value of *CD200* in CSCC. After 5-year follow-up, Kaplan-Meier analysis of the survival rate of patients with CSCC in our study indicated that patients with low *CD200* expression had a longer survival compared to those with high expression (log-rank test, $P<0.001$). A multivariate analysis based on univariate analysis validated the prognostic value of *CD200* expression adjusted for the clinicopathologic characteristics, and indicated that *CD200,* as well as differentiation grade and clinical stage, could serve as independent predictors for the prognosis of CSCC.

Conclusions

The expression level of *CD200* in tumor tissues is up-regulated compared to that in adjacent normal tissues. Its expression is significantly affected by tumor differentiation grade and clinical stage. Furthermore, the over-expression of *CD200* is related to shorter survival time of patients with CSCC. It seems that *CD200* is a potential prognostic predictor in CSCC.

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