Int J Clin Exp Pathol 2014;7(5):2470-2476
www.ijcep.com /ISSN:1936-2625/IJCEP0000133

Original Article

Embryonic stem cell markers Sox-2 and OCT4 expression and their correlation with WNT signal pathway in cervical squamous cell carcinoma

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Received February 28, 2014; Accepted April 26, 2014; Epub April 15, 2014; Published May 1, 2014

Abstract: Background: Both the expression of embryonic stem cells (ESCs) markers (Sox2, Oct4) and the Wnt signal pathway (β-catenin) are crucial for progression of various human malignancies. The purpose of this study was to investigate the clinicopathologic significance of Sox2, Oct4 and β-catenin in cervical squamous cell carcinoma (CSCC) and to study their correlation with the occurrence and prognosis. Methods: Sox2, Oct4 and β-catenin were assessed using immunohistochemistry in normal cervix tissues (n = 28) and invasive cervical squamous cell carcinoma (n = 43). Associations of Sox2, Oct4 and β-catenin levels with clinicopathological characteristics and with overall survival were studied using uni- and multivariate analysis. Results: The expression levels of Sox2, Oct4 and β-catenin were highly increased in CSCC compared with the normal cervix tissues. The ESCs markers expression (Sox2 and Oct4) correlated significantly with β-catenin expression. High expression of Sox2, but not that of Oct4 or β-catenin, was correlated with poorer differentiation ($P < 0.05$). Furthermore, Sox2 expression was significantly correlated with patients' status of survival in advanced CSCC ($P < 0.05$), whereas there was no significant finding in Oct4 or β-catenin expression. Conclusions: These findings provide evidence that both ESCs biomarkers (Sox2, Oct4) and Wnt signal pathway (β-catenin) are activated in CSCC. Sox2 can be regarded as a novel predictor of poor prognosis for CSCC patients.

Keywords: Sox2, Oct4, β-catenin, immunohistochemistry, cervical squamous cell carcinoma, prognosis

Introduction

Cervical cancer is the second-leading cause of cancer deaths in women worldwide, and results in approximately 270,000 deaths each year [1]. Most cervical carcinomas are related to human papilloma virus (HPV) in etiology and have a stepwise progression starting from premalignant lesions; however, HPV is not sufficient for cervical carcinogenesis and tumor progression [2, 3]. Recent studies have revealed the critical role of cancer stem cells (CSCs) in tumorigenicity and metastasis [4]. To date, CSCs have been identified in numerous solid cancers, such as breast cancer, neuroblastoma, colon cancer and lung cancer [5-8], as well as cervical cancer [9].

Sox2 (SRY-related HMG-box gene 2), is a key transcription factor to maintain the self-renewal capability and pluripotency of embryonic stem cells (ESC). Oct4 (Octamer 3/4), which belongs to the family of POU-domain transcription factors, is also required for self-renewal and differentiation potential of pluripotent ESC. Of interest, recent studies indicated these stem cell markers might have emerged in the process of tumorigenesis [10]. The mechanism of that process remained unknown.

The canonical Wnt signal pathway has emerged as a critical regulator of stem cells in many tissues [11]. Dysregulation of the Wnt signal pathway has also been implicated in several tumorigenesis, such as colorectal cancer [12]. The presence or absence of some members in this pathway, such as β-catenin, APC, has been found to involve in different types of tumors. However, the correlations of the stem cells markers and Wnt signal pathway with clinico-

pathological features in cancers still remain poorly understood.

In this study, we used immunohistochemistry (IHC) to assay the expression of stem cell markers (Sox2, Oct4) and crucial gene of Wnt signal pathway (β-catenin) in 43 human cervical squamous cell carcinoma (CSCC) and 28 cases of normal cervix. The correlation between the expression of stem cell markers and Wnt signal pathway, and their correlation with clinicopathological factors, prognosis, were analyzed as well.

Materials and methods

Patients and specimens

The present study was approved by the institutional review board named as "Ethics Committee of Medical School of Xi'an Jiaotong University" in Shannxi, China. Ethics Committee of Medical School of Xi'an Jiaotong University approved the design of cervical cancer study including tissue samples collection, follow-up.

All biopsies of 43 CC patients and 28 normal cervix tissues were collected from the Department of Gynecology and Radiation Oncology, between 2011 and 2012. All the patients were diagnosed with pathologic examination. None of the patients had received preoperative radiotherapy or chemotherapy. The clinical stage of the patients was classified according to the International Federation of Gynecology and Obstetrics (FIGO) criteria as follows: 14 cases were allocated to stage I, 20 to stage II, 9 to stage III and stage IV. Mean patients age was 49.6 (range from 29 to 71).

Immunohistochemistry (IHC)

IHC analysis was performed to examine the protein expression of Sox2, Oct4, and β-catenin. All immunohistochemical staining were done using 4 μm serial tissue sections. They were first deparaffinized and rehydrated with a gradient concentration of alcohol. Endogenous peroxidase activity in the sections was block by 3% H_2O_2. Then the sections were subjected to high pressure for antigenic retrieval for 2 min. The slides were incubated overnight at 4°C with primary antibodies used as follows: Sox2 (clone Y-17, 1:50, Santa Cruz); Oct4 (No. 2750, 1:100, Cell Signaling Technologies, CST; clone C-20, 1:100, Santa Cruz); β-catenin (No. 9582, 1:100, CST); or phosphate buffered saline (PBS) as negative control. After washing, the sections were incubated with prediluted secondary antibody for 30min and the staining was visualized with DAB. Finally, the slides were counterstained with hematoxylin and mounted in an aqueous mounting medium.

Scoring of immunohistochemistry

All stained slides were evaluated by two independent investigators without knowledge of the clinicopathological data. For the evaluation of IHC results, staining intensity (SI) was assessed as follows: 0, no staining; 1, weak staining; 2, modest staining; 3, strong staining. Likewise, the proportion of tumor cell staining (P) was evaluated by four grades: 0, < 10% positive tumor cells; 1, 10%-25% positive tumor cells; 2, 26%-50% positive tumor cells; 3, 51%-75% positive tumor cells; 4, > 75% positive tumor cells.

For Sox2, only nuclear staining was considered as positive, the results were evaluated by multiplying SI with P (minimum 0/maximum 12). Staining scores < 4 and ≥ 4 were regard as tumors with low and high expression. For β-catenin and Oct4, a strong and disuse cytoplasmic staining was considered as positive in cells, and the results was analyzed only by SI. For statistical analysis the cases were classified into low (0-1) and high (2-3) grade expression.

Follow-up and statistical analysis

Follow-up information was available for all the patients with a median time of 25.56 months (range 12-34 months). All patients were monitored by physical examination, ultrasonography and laboratory analysis. All statistical analyses were carried out using SPSS 18.0 (SPSS Inc, Chicago). Qualitative data was analyzed by χ^2 test or Fisher's exact test, while the quantitative data was analyzed by Wilcoxon rank sum test. Correlations between variable factors were estimated by Spearman correlation coefficients. Only $P < 0.05$ was considered to be statistically significant.

Results

Expression of Sox2 and Oct4 in CSCC tissues

To investigate the expression of ESCs-related markers in CSCC, we performed immunohisto-

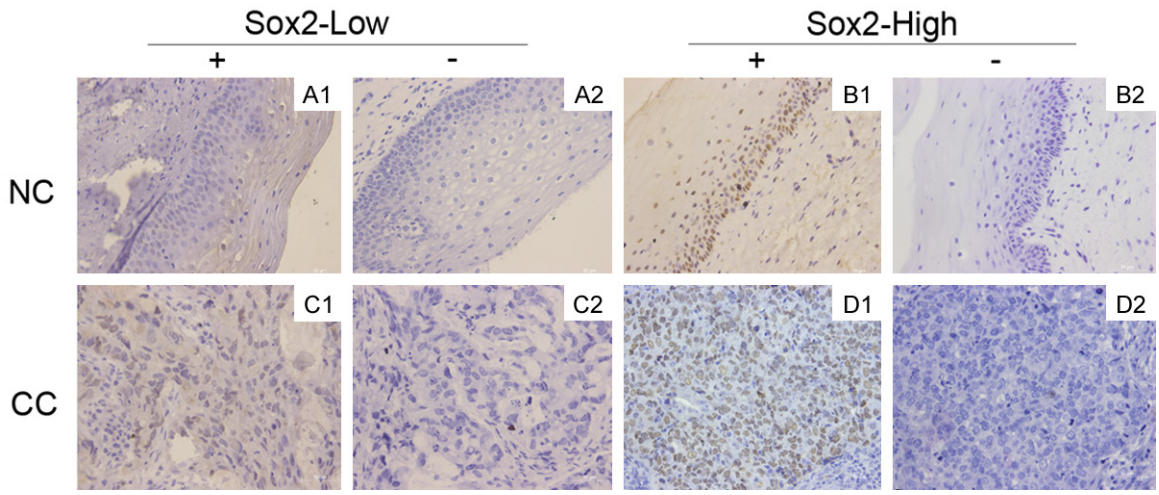

Figure 1. Immunohistochemical staining of Sox2 in NC and CC. Sox2 was low expressed or highly expressed in normal cervix (A, B) and cervical squamous cell cancers (C, D).

Table 1. Expression of Sox2, Oct4 and β-catenin in tissues of CSCC and NC

Group	Case	Sox2		*P*	Oct4		*P*	β-Catenin		*P*
		Low	High		Low	High		Low	High	
CC	43	18	25	0.000**	19	24	0.024*	17	26	0.000**
NC	28	24	4		20	8		25	3	
Total	71	42	29		39	32		42	29	

CSCC: cervical squamous cell carcinoma; NC: normal cervix. *$P < 0.05$; **$P < 0.01$.

chemical analyses with Sox2- and Oct4-specific antibody in 43 cases of human cervical squamous cell cancers and 28 cases of normal cervix. No positive of Sox2 protein was detected in normal cervix, only 4 cases (14.3%) exhibited the nuclear staining in basal cells layers (**Figure 1A**). In contrast, 25 samples (58.1%) of the CSCC showed high nuclear expression of Sox2 ($P < 0.001$, **Table 1**).

Interestingly, the specific Oct4 staining was localized mainly in the cytoplasm of tumor cells as brown staining (**Figure 2D1**). High expression of Oct4 was found in 24 cases (55.81%) of CSCC, whereas only 8 cases (28.5%, **Figure 2B1**) showed high expression in the basal cell layers of normal cervix ($P = 0.024$).

β-catenin protein was expressed in CSCC

In normal epithelia, immunoreactivity was observed in most cases at the plasma membrane of both basal and parabasal cells, only 3 cases (3/28, 10.7%) were expressed in the cytoplasm (**Figure 2A1**). No immunoreactivity was observed in superficial layers of normal epithelium. On the other hand, strong cytoplasmic expression of β-catenin was detected in 60.5% (26/43) of CSCS ($P < 0.001$, **Figure 2C**). Interestingly, absent immunostaining at the membrane was observed in a large number of CSCC.

Correlation between Sox2, OCT4 and β-catenin

Since both the transcription factors of ESCs and Wnt signal pathway play a crucial role in cancers' growth and metastasis, we assessed the correlation of Sox2 and Oct4 with the expression of β-catenin in CSCC. As shown in **Table 3**, a significant association was identified between the expression of Sox2 and β-catenin ($r = 0.567$, $P < 0.001$). Similarly, high expression of Oct4 was significantly associated with increased β-catenin expression ($r = 0.334$, $P = 0.029$), whereas there was no significant correlation between Sox2 and Oct4 ($P > 0.05$).

Associations of the clinicopathological features with Sox2, Oct4, β-catenin expression

The association between Sox2, Oct4 and β-catenin expression and clinicopathological characteristics of CSCC in summarized in **Table**

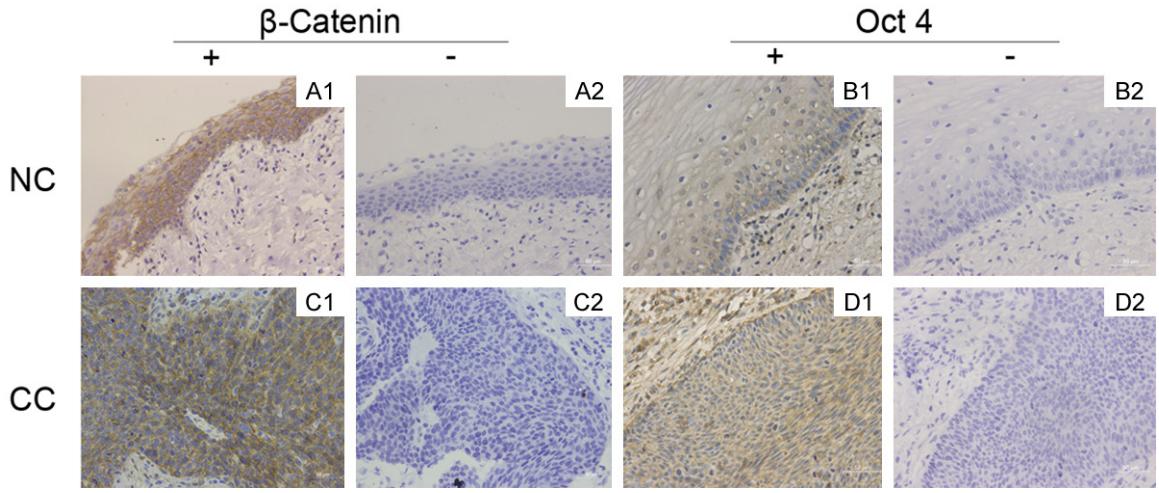

Figure 2. Expression of β-catenin and Oct4 in NC and CC. (A) β-catenin was positive in NC (A1) and negative control (A2). (B) Oct4 was positive in NC (B1) and negative control (B2). (C) β-catenin was positive in CC (C1) and negative control (C2). (D) Oct4 was positive in CC (D1) and negative control (D2).

Table 2. Expression of Sox2, Oct4 and β-catenin of clinicopathological features in CSCC

Features	No. patients	Sox2		*P*	Oct4		*P*	β-Catenin		*P*
		Low	High		Low	High		Low	High	
Histological grade										
I-II	23	11	12	0.049*	10	13	0.872	11	12	0.319
III	20	7	13		9	11		6	14	
Clinical stages (FIGO stages)										
I	14	5	9	0.877 (1, 2)	8	6	0.478 (1 ,2)	6	8	0.616 (1, 2)
II	20	8	12	0.871 (2, 3)	8	12	0.799 (2, 3)	8	12	0.764 (2, 3)
III-IV	9	5	4	1.000 (1, 3)	3	6	0.369 (1, 3)	3	6	0.477 (1, 3)
Lymph node metastasis										
Yes	9	3	6	0.355	6	3	0.112	4	5	0.848
No	25	10	15		9	16		10	15	
Age (years)										
> 49	23	8	15	0.313	9	14	0.474	9	14	0.954
≤ 49	20	10	10		10	10		8	12	

*P < 0.05.

2. None of the Sox2, Oct4, β-catenin expression was correlated to age, lymph node status and tumor/FIGO stage. However, high levels of Sox2 expression was significantly associated with poorer differentiation of CSCC ($P < 0.05$), whereas Oct4 and β-catenin did not have this correlation.

Correlation between prognosis of CSCC and the expression of Sox2, Oct4, β-catenin

We investigated the correlation between the prognosis of CSCC and the expression of Sox2, Oct4 and β-catenin. Among all 43 CSCC patients, 9 cases (stage III and stage IV) who had not undergone surgical operation treatment were analyzed (**Figure 3A**). The expression of Sox2 in the group of patients who had died during follow-up period was higher than those still survived (**Figure 3B**), the difference between two groups was significant ($P = 0.032$). However, no significant correlation was observed between Oct4 or β-catenin expression and overall survival of patients ($P > 0.05$, data not show).

Discussion

In the present study, we analyzed the expression of several proteins commonly considered

Table 3. Relationship of Sox2, Oct4 and Wnt signal pathway in CSCC

Sox2	β-Catenin		*r*	*P*	Oct4		*r*	*P*	Oct4	β-Catenin		*r*	*P*
	Low	High			Low	High				Low	High		
Low	13	5	0.567	0.000**	10	8	/	0.212	Low	11	8	0.334	0.029*
High	4	21			9	16			High	6	18		
Total	17	26			19	24			Total	17	26		

*$P < 0.05$; **$P < 0.01$

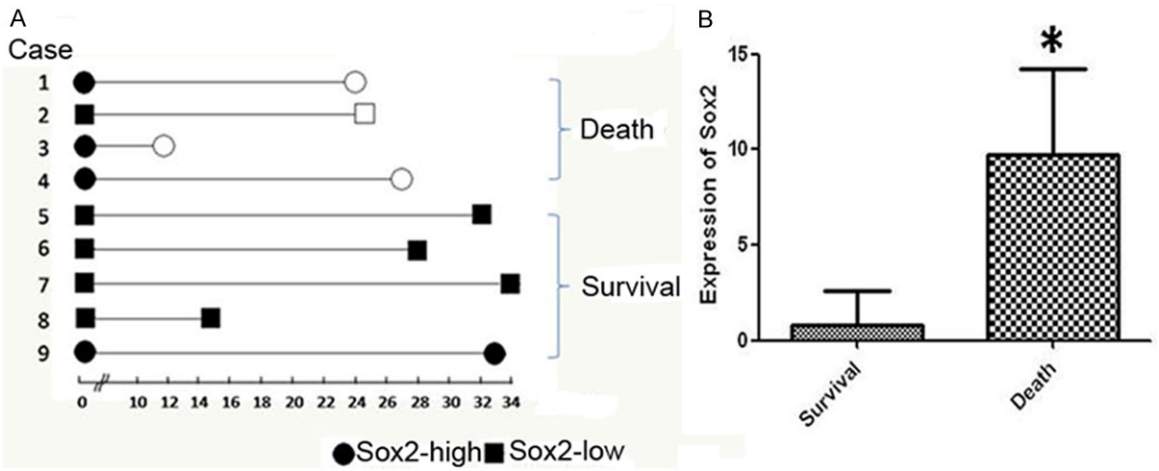

Figure 3. Follow ups of the patients (Stage III-IV) and analysis of the expression of Sox2. A. Prognosis of patients with advanced CSCC (Stage III-IV, 9 cases) was analyzed. The median follow-up period was 25.6 months (range 12-34 months). Black diamonds indicate Sox2 was high expression, black squares indicated Sox2 was low expression. B. Sox2 expression was higher in the patients who were dead during the follow-up than those were still survival ($P = 0.032$).

as ESCs markers in human CSCC specimens and found associations with clinicopathological features, as well as correlations between ESCs markers and the Wnt signal pathway.

Embryonic stem cells (ESCs) are defined as cells that have ability to self-renew, pluripotency and to differentiate into a variety of cell types [13]. It is generally considered that Sox2, Oct4, and Nanog are key transcription regulators of ESCs. Recently, the crucial transcription factors have also been recognized as having "stemness" characteristics in cancer cells, and contributed to carcinogenesis, tumor metastasis and poor outcome [14, 15]. However, little is known about the expression levels of these molecules and their correlations with clinical significance in CSCC patients. Compared with normal cervix tissues, we observed that both Sox2 and Oct4 protein expression were highly increased in cervical squamous cell carcinoma tissues, suggesting that Sox2 and Oct4 might be involved in the pathogenesis of CSCC. A statistical analysis clearly demonstrated the significant association of the Sox2 expression with poorer differentiation. Furthermore, patients with Sox2 high-expression had significantly worse overall survival. These results were consistent with the finding in several malignances including breast cancer [16], bladder cancer [17], and colon cancer [18]. In small cell lung cancer, some studies demonstrated that the expression of Sox2 was also significantly associated with late clinical stage and lymph node metastasis [19]. However, such a relation did not observe in our study, probably because of the restricted number of cases. The data presented here need to be confirmed in a larger cohort of advanced cervical cancer cases with various grades and lymph node metastasis.

The transcription factor Oct4 is an important determinant for the malignant potential of tumor cells and can be detected in human embryonic carcinomas, testicular germ cell

tumors, and glioma cells [20, 21]. Knocking down Oct4 in tumor-initiating cells would lead to the loss of the self-renewal and proliferation capacities and result in CSC-like apoptosis of cancer cells [22]. In CSCC, Oct4 expression was identified in more than one half of cases. However, the immunohistochemical analysis showed the specific Oct4 staining was localized in the cytoplasm of tumor cells, not in the nucleus as previous reported [14]. Furthermore, it did not correlate with clinical stage, tumor stage or lymph node metastasis. It is well documented that Oct4, Sox2 and Nanog are three core transcription factors maintain the potency and self-renewal of ECSs, and that they are all localized in the cell nuclei. The function of ECS-associated proteins in the cytoplasm remains to be determined. One possible explanation for this would be that Oct4 has many pseudogenes and spliced variants, and this has led to confusion in stem cell research and current knowledge of these genes in cancers [23].

The canonical Wnt/β-catenin signaling plays a substantial role in self-renewal, pluripotency, proliferation and cell-fate determination of ESCs. Recently, it has been found that Wnt/β-catenin signaling is one of the key pathways in the maintenance of CSCs (for example in lung, colon, liver, breast cancer) [24-26]. In our study, we found a striking cytoplasmic localization of β-catenin in the majority of the cases (60.5%). This data is in agreement with previous studies [27], and suggest that the presence of β-catenin in the cytoplasm may be related to the malignant phenotype of epithelial cells from the uterine cervix. Our data showed that the expression of β-catenin was not related to the degree of histologic differentiation, clinical stage and lymph node metastasis, this might also probably because of the restricted number of cases. There are some controversial reports on the association of β-catenin expression in CSCC with the clinicopathological features; therefore, further studies with a large patient population are necessary to explore the role of β-catenin with standard methods for different types of cervical cancers.

In this study, we found that both the high expression of Sox2 and Oct4 were significantly associated with increased β-catenin expression. To our knowledge, this is the first study to show that the association between ESCs markers and the Wnt signal pathway. The expression of Sox2 was significantly associated with overall survival by univariate analysis, whereas there were no significant associations with overall survival in multivariate Cox analysis. Thus, the present study has some limitations. First, the patients with cervical cancer in stage III or IV were only 9. Second, early-stage cervical squmaous cell cancer has a good prognosis, more than 5-years of post treatment surveillance without evidence of recurrence [28].

In summary, the current study revealed for the first time that Sox2, Oct4 and β-catenin were overexpressed in CSCC as compared with normal tissues by IHC, and there were significant associations between Sox2, Oct4 and β-catenin expression. We also postulate that Sox2, but not Oct4, might be used as a prognostic biomarker and also a new target for CSCC. Indeed, further investigations should be required to illustrate the mechanism of Sox2 and β-catenin in CSCC.

Acknowledgements

This work was supported by the National Science Youth Foundation of China (No. 81001162).

Disclosure of conflict of interest

None.

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