



***Wnt1* and *SFRP1* as potential prognostic factors and therapeutic targets in cutaneous squamous cell carcinoma**

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ABSTRACT. The Wnt signaling pathway plays a key role in insurgence and progression of many different forms of cancer. Some crucial components of the Wnt pathway have been proposed to be novel targets for cancer therapy. To date, the Wnt signaling pathway has not been studied in cutaneous squamous cell carcinoma (CSCC). This study was designed to investigate the expression of *Wnt1* and *SFRP1* from the Wnt pathway in CSCC. Tissue samples were obtained from 35 patients with CSCC and 30 controls admitted to the Xinjiang

Uyгур Autonomous Region People's Hospital at Urumchi City, China. Gene and protein expressions of *Wnt1* and *SFRP1* were quantified by immunohistochemistry and western blotting. *Wnt1* expression was significantly higher ($P < 0.05$) in CSCC samples than in normal skin cells of the control subjects; in contrast, *SFRP1* expression was significantly lower in CSCC tissues than that in tissues of control subjects ($P < 0.05$). Moreover, *Wnt1* expression ($P < 0.05$) was found to be correlated with histopathological differentiation in CSCC, and negatively correlated with *SFRP1* expression in CSCC ($r_s = -0.473$, $P = 0.015$). Therefore, we concluded that *Wnt1* and *SFRP1* play important roles in the development of CSCC and could be potent markers for diagnosis, prevention, and therapy of CSCC.

Key words: *Wnt1*; *SFRP1*; Cutaneous squamous cell carcinoma

INTRODUCTION

A sequence of transitional events, such as hyperplasia, dysplasia, papilloma, and cutaneous squamous cell carcinoma, are involved in skin carcinogenesis (CSCC) (Calzado et al., 2007). Some gene products have been shown to influence cell proliferation and control the cell cycle; therefore, these genes and their protein products could serve as targets for drug delivery and therapy. Additionally, these genes and proteins may also be used as prognostic markers (Grimm et al., 2013). Therefore, further knowledge of the molecular signaling pathways actively involved in CSCC insurgence and progression could provide new targets for new therapeutic strategies with better prognoses than those that are currently available.

A number of studies conducted over the past decade have determined the crucial role of deregulation or constitutive activation of the Wnt signaling pathway in carcinogenesis and the neoplastic transformation of different forms of human cancer, including breast cancer (Holland et al., 2013), colorectal cancer (Spreafico et al., 2013), gastric cancer (Tang et al., 2013), and prostate cancer (Jiang et al., 2013). The Wnt protein family is a family of secreted glycoproteins that regulate cell proliferation, differentiation, migration, and apoptosis, therefore playing a key role in embryo development and maintenance of homeostatic balance in humans (Chang et al., 2004). The Wnt signaling pathway is regulated by several secreted inhibitors, such as the secreted frizzled-related protein 1 (*SFRP1*). *SFRP1* belongs to a family of secreted glycoproteins (*SFRP1-SFRP5*) that function as modulators of the Wnt signaling pathway, while playing a role in the development of cancer (Liu et al., 2015). The SFRP family possesses structural similarities to the cysteine-rich domain of frizzled receptors, allowing it to interact with Wnt to form the Wnt-SFRP complex. Through this interaction, the Wnt protein is sequestered from the frizzled receptor (Bhanot et al., 1996).

CSCC is currently the second most-common human skin cancer after basal-cell carcinoma (Bouwes Bavinck et al., 2010). However, very few studies have analyzed the role of the Wnt signaling pathway in CSCC, particularly in carcinogenesis. In this study, we investigated the role of *Wnt1* and *SFRP1* expression in patients with CSCC using immunohistochemistry and western blotting. Elucidating the specific molecular and functional changes that underlie the development of CSCC may help us in developing optimum treatment strategies and better understand the role of the Wnt signaling pathway in skin development and function.

MATERIAL AND METHODS

Participants and tissue samples

Thirty-five patients newly diagnosed with histopathologically confirmed CSCC were recruited between January 2012 and February 2014 from the Dermatology Department of the Xinjiang Uygur Autonomous Region People's Hospital at Urumchi City, China. None of the patients has been previously diagnosed with other types of cancer or undergone chemotherapy or radiotherapy. Thirty age- and gender-matched controls with no history of CSCC were selected from the Department of Plastic Surgery of the same hospital. *Wnt1* and *SFRP1* expression was analyzed in CSCC and normal tissue samples taken from 35 patients and 30 controls, respectively. The relevant clinical data of all participants are listed in Table 1.

Table 1. Clinical data of the participants.

	Case	Control
Gender		
Male	20	16
Female	15	14
Age (years)		
Range	49-92	39-90
Mean	67.54 ± 8.84	65.35 ± 8.48
Disease duration (months)		
Range	6-180	
Mean	23.05 ± 1.47	
Grade		
CSCC I	12 (34.28%)	
CSCC II	15 (42.85%)	
CSCC III	8 (22.85%)	
CSCC IV	0	

CSCC = cutaneous squamous cell carcinoma.

Tissue samples and signed informed consent forms were obtained from all participants. This protocol was approved by the Clinical Research Ethics Board of the Xinjiang Uygur Autonomous Region People's Hospital, and is in compliance with the Department of Health and Human Services (DHHS) regulations for the protection of human research subjects.

Immunohistochemistry staining

Immunostaining of *Wnt1* and *SFRP1* was performed on parallel histopathological sections from paraffin-embedded CSCC and normal control tissue sections. The tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min, and subsequently washed three times with phosphate-buffered saline (PBS, pH 7.4) for 5 min each (Bogoo Biotechnology Company, Shanghai, China). Tissue sections were incubated overnight at 4°C with primary monoclonal *Wnt1* and *SFRP1* antibodies. The samples were washed with PBS and incubated for 30 min at room temperature with a biotinylated secondary antibody. Immunolabeling was visualized by incubating the sections with 3,3'-diaminobenzidine for 10 min. The sections were washed and counterstained with hematoxylin and observed under a light microscope (Olympus BH-2, Tokyo, Japan). The primary antibody was replaced with PBS in negative controls.

The immunohistochemistry scoring results for *Wnt1* and *SFRP1* were based on the staining intensity and fraction of immunopositive cells. Positive Wnt expression was defined by distinct brown cytoplasmic staining. SFRP1 expression was identified by distinct brown cytoplasmic and nuclear staining. The intensity of nuclear staining was scored as follows: 0, no expression; 1+, mild expression; 2+, moderate expression; and 3+, strong expression. The percentage of positive cells was determined by evaluating the entire lesion on the slide. The mean percentage of positive cells was determined for each marker, for five fields per section at high magnification, and was assigned to one of the following categories: 0, 0%; 1+, <25%; 2+, 25-50%; 3+, 51-75%; and 4+, >75%. The scores indicating the percentage of positive cells and staining intensity were multiplied to produce a semi-quantitative immunohistochemical score: 0-2 points = negative (-); >2 points = positive (+).

Western blotting

Cells were harvested and lysed with a buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, and the protease inhibitors aprotinin, leupeptin, and phenylmethanesulfonyl fluoride (PMSF). The cellular protein was loaded onto a 5-12% gradient SDS-PAGE precast gel and transferred onto a nitrocellulose membrane in Tris-buffered saline-0.05% Tween-20 (TBST). Primary antibodies against *Wnt1* and *SFRP1* were used to detect endogenous protein expression. Horseradish peroxidase-labeled secondary antibodies were detected using a chemiluminescence reagent and photographic film. *GAPDH* was used as the positive control.

Statistical analysis

The data were statistically analyzed using Microsoft Excel and SPSS v.16.0 (SPSS Inc., Chicago, IL, USA). The data were assessed using the Student *t*-test; P values = 0.05 were considered to be statistically significant.

RESULTS

Wnt1 and SFRP1 immunohistochemical expression

Morphologically normal skin from control subjects was negative for *Wnt1* immunostaining, while corresponding CSCC samples showed moderate to strong positive cytoplasmic staining. In contrast, *SFRP1* expression was observed in normal skin, and not in the cancer cell nests (Figure 1).

Positive Wnt1 signals were also observed in 20 (20/35; 57.14%) CSCC cases and 7 (7/30; 23.33%) control samples. However, *Wnt1* expression was significantly higher in CSCC cells than that seen in the normal group ($P < 0.05$). Moreover, patients with CSCC III showed significantly higher expression of *Wnt1* than those with CSCC I ($P < 0.05$). Eight (8/35; 22.86%) patients with CSCC and 20 (20/30; 66.67%) control subjects showed *SFRP1* staining; moreover, *SFRP1* expression was significantly lower in CSCC samples than that in the normal control samples ($P < 0.05$). However, the positive *SFRP1* staining rates did not differ significantly between the different CSCC groups (Table 2).

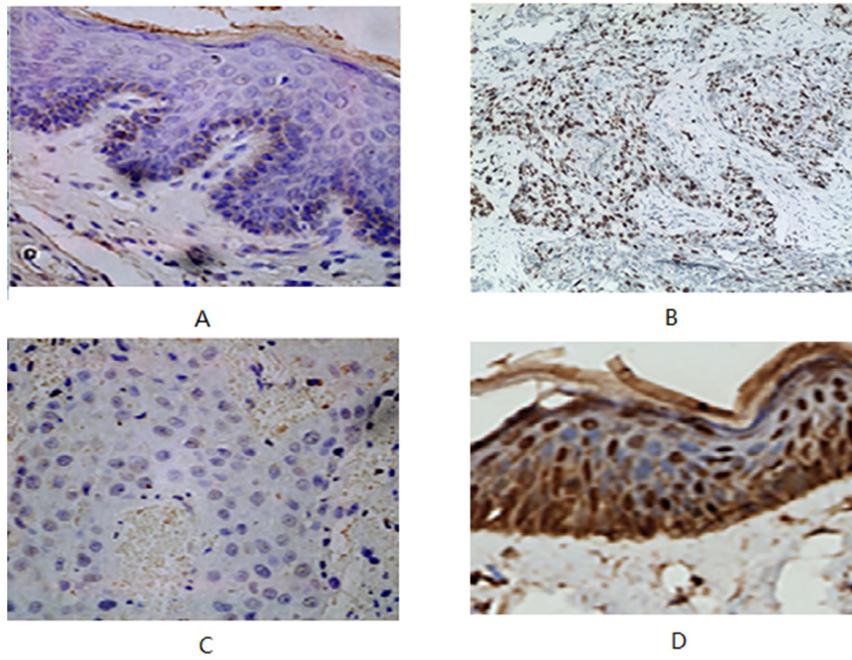


Figure 1. Immunohistochemical staining for *Wnt1* and *SFRP1* in cutaneous squamous cell carcinoma (CSCC) and normal skin tissues (magnification 100X). **A.** Negative expression of *Wnt1* in normal skin tissue. **B.** Positive expression of *Wnt1* in CSCC tissues. **C.** Negative expression of *SFRP1* in CSCC. **D.** Positive expression of *SFRP1* in normal skin tissues.

Table 2. Expression of *Wnt1* and *SFRP1* in cutaneous squamous cell carcinoma (CSCC) and normal skin tissue and its relationship with pathological features of CSCC.

Group	N	<i>Wnt1</i>		Positive rate (%)	χ^2	P	<i>SFRP1</i>		Positive rate (%)	χ^2	P
		+	-				+	-			
CSCC	35	20	15	57.14	7.604	<0.05	8	27	22.86	12.644	<0.05
Normal	30	7	23	23.33			20	10	66.67		
CSCC I	12	5	7	41.67	<0.05	<0.05	3	9	10.83		>0.05
CSCC II	15	9	6	53.33			4	11	26.67		
CSCC III	8	6	2	75.00			1	7	12.50		

CSCC samples showed high expression of *Wnt1* and low expression of *SFRP1*; therefore, we calculated the correlation between *Wnt1* and *SFRP1* expression in the CSCC and control groups. We observed a negative correlation in the CSCC group ($r_s = -0.473$, $P = 0.015$), and no correlation in the control group ($r_s = -0.901$; $P = 0.069$) (data not shown).

Western blot analysis for *Wnt1* and *SFRP1*

The western blot results were consistent with the immunostaining results. *Wnt1* protein levels were higher in the CSCC group than in the control group. In contrast, *SFRP1* levels were higher in the control group. These differences in *Wnt1* and *SFRP1* expression were statistically significant ($P < 0.05$; Table 3).

Table 3. Western blot results of *Wnt1* and *SFRP1* expression in cutaneous squamous cell carcinoma (CSCC) and normal skin tissues.

Group	N	<i>Wnt1</i>	<i>SFRP1</i>
CSCC	35	0.829 ± 0.024	0.231 ± 0.029
Control	30	0.176 ± 0.020	0.809 ± 0.214
<i>t</i>		4.891	4.567
<i>P</i>		0.008	0.010

DISCUSSION

The quality and accuracy of currently available (and proposed) biomarkers for early CSCC diagnosis, staging, and prognosis is poor; therefore, there is an urgent need for absolute positive and negative immunohistochemical markers. However, the limited research into specific CSCC markers has shown that the identification of specific positive or negative indicators could allow for the simultaneous detection of multiple immunohistochemical markers for CSCC. Recent years have seen an increase in research into the molecular mechanisms that underlie Wnt signaling; in fact, pharmaceutical and biotechnological companies have invested a significant amount of resources into this research, as drugs targeting this pathway could be very useful for the development of chemopreventive strategies for CSCC (Moon et al., 2004; Patel et al., 2008; Anastas and Moon, 2013). As the level of expression of *Wnt1* and *SFRP1*, and their subcellular localization, could be indicative of Wnt signaling pathway activation, we analyzed these genes and their resultant proteins in this study.

Wnt1 was highly expressed in CSCC patient tissue samples. Nusse et al. (1984) first identified the role of Wnt proteins in mouse models of mammary cancer in 1982, and reported that aberrant overexpression of *Wnt1* could lead to spontaneous mammary hyperplasia and tumors in mice. Additionally, Rhee et al. (2002) reported a significant increase in the levels of Wnt1 proteins in ten head and neck squamous carcinoma cell lines compared to normal epithelial cells, which was in agreement with our findings. We also observed a significant increase in *Wnt1* expression with the increase in histological differentiation, suggesting the important role played by *Wnt1* in the development of CSCC. Therefore, we concluded that this could be used as a prognostic marker.

There are two types of factors that inhibit Wnt signaling: SFRP and Dickkopf (DKK) proteins (Oshima et al., 2005; Aguilera et al., 2006). In this study, the expression of *SFRP1* in CSCC tissues was found to be significantly lower than that seen in normal skin samples. Moreover, it was observed that *Wnt1* and *SFRP1* expressions were negatively correlated in CSCC tissue. These data suggested that *Wnt1* and *SFRP1* play a competitive role in CSCC progression, which must be confirmed in further studies.

In conclusion, we demonstrated that crucial components of the Wnt signaling pathway, *Wnt1* and *SFRP1*, function as potent markers for the diagnosis, prevention, and therapy of CSCC, using immunohistochemistry and western blot analysis. We believe that these markers could be used to predict the responsiveness of patients with CSCC to Wnt-targeting therapy.

Conflict of interest

The authors declare no conflict of interest.

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