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Original Article

SFRP2 DNA hypermethylation and decreased protein expression of ESCC in Kazakh and Han patients

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Abstract: Background: Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies in China, particularly in northwest area. However, no effective biomarkers have been identified for the diagnosis of patients with ESCC. It has been reported that Wnt signaling pathway plays an important role in Esophageal Cancer. Secreted frizzled-related protein 2 (SFRP2) is a further Wnt inhibitor whose expression was recently found being down-regulated in various malignancies, and its underlying mechanism are poorly understood. Here, our aim of the study was to investigate the expression levels and methylation status of the SFRP2 in ESCC, and to evaluate the clinical utility of the marker. Methods: In this study, we used immunohistochemistry (ICH) to measure the expression levels of SFRP2 in ESCC tumor tissue and the corresponding matched adjacent normal tissue. Methylation-special polymerase chain reaction (MSP) was employed to evaluate the aberrant methylation status of SFRP2 in DNA. Then, the correlation between SFRP2 protein expression and SFRP2 promoter methylation with clinicopathological parameters multiethnic differences and prognosis outcomes of ESCC patients were statistically analyzed respectively. Results: SFRP2 protein expression increased progressively from normal esophageal epithelium to ESCC. SFRP2 promoter methylation was detected in ESCC tumor tissues, whereas, a little normal tissues were affected by SFRP2 methylation. Of ESCC tumor tissues, most patients' tissues lacked SFRP2 protein expression due to SFRP2 promoter methylation. Conclusion: The present analysis demonstrated that SFRP2 may play an important role in the carcinogenesis and progression in terms of promoter methylation and protein expression.

Keywords: Esophageal squamous cell carcinoma, secreted frizzled-related protein 2, methylation, immunohistochemistry, clinicopathologic factors

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed malignant tumors remaining predominant histological type of esophageal cancer, which has a very poor prognosis in China [1-3], especially in the Chinese Kazakh ethnic population residing in Xinjiang, northwest china [4]. In Xinjiang, Kazakh ethnic people were easily suffered cancer, due to the nitrosamine-rich or mycotoxin-contaminated foods and low socioeconomic status [5]. ESCC is a highly lethal cancer, and ranks the sixth most common cause of cancer-related death worldwide, the seventh incidence in China [6]. However, despite the improvements of its diagnosis and multimodal treatment, its underlying molecular mechanisms are poorly understood [7]. Therefore, it is eagerly to identify the underlying molecular markers as new prognostic and therapeutic targets in ESCC diagnosis and treatment respectively in Kazakh and Han ethnic population.

The Wnt signaling pathway involved not only in embryonic development and tissue differentiation, but also in cancer progression [8]. The Wnt signaling pathway can be partially regulated by Wnt antagonists, including members of the Dickkopf family, Wnt inhibitory factor and secreted frizzled-related proteins (SFRPs). The SFRPs constitute a family of extracellular Wnt signaling antagonists, of which five members (SFRP1-5), each containing a cysteine-rich domain.

SFRPs are a family of secreted glycoproteins, which are recognized as negative regulators of the Wnt signaling pathway.

A previous study confirmed that aberrant promoter methylation of secreted frizzled-related protein 2 (SFRP2) gene is important in the progression of several types of human tumors, such as colorectal cancer [9], gastric cancer [10], and oral squamous cell carcinoma [11], suggesting that SFRP2 is a tumor suppressor. Based on analysis of the SFRP2 gene promoter methylation status and the expression levels of SFRP2 protein in cancer tissues and adjacent non-cancer tissues from ESCC patients, the present study investigated the effects of SFRP2 on the Wnt signaling pathway in the development of ESCC.

Materials and methods

Patients and tissue samples

Between 2007 and 2015, 100 patients with ESCC were enrolled in this study from the First Affiliated Hospital of Xinjiang Medical University, who underwent resection for ESCC without radiotherapy or chemotherapy prior to the surgery. The patients consisted of 50 Kazakhs and 50 Han patients, normal esophageal epithelial samples were taken from the corresponding normal mucosa, as non-tumor control samples. Diagnosis was performed at the Department of Pathology, First Affiliated Hospital of Xinjiang Medical University. All of the 100 formalin-fixed, paraffin-embedded samples were histopathologically diagnosed as ESCC. Ethical approval was obtained from the ethics committee of the First Affiliated Hospital of Xinjiang Medical University. Informed consents were obtained from all participants. Tumor stage was determined according to the American Joint Committee on Cancer (AJCC), involved tumor size (T), lymph node involvement (N), and distant metastasis (M). Then, the following patient characteristics were collected for the 100 ESCC patients, including: age, gender, tumor location, tumor size, degree of differentiation, ethnic, clinicopathological stage, lymph node status. Clinical follow-up information was obtained by telephone or from the outpatients' records.

Immunohistochemistry

Paraffin-embedded 3 μm sections were baked at 65°C for 120 minutes, then deparaffinized in xylene for 30 minutes and dehydrated in graded ethanol for 5 minutes. The sections were autoclaved in 1% sodium citrate buffer (PH 6.0), cooled at room temperature. Then the slides were incubated with fresh 3% H_2O_2 in methanol for 15 min at room temperature to quench the endogenous peroxidase activity. Tissue sections were then incubated at 4°C overnight carrying out anti-SFRP2 rabbit polyclonal antibody at a dilution of 1:100. After washed in PBS, the slides were incubated with corresponding secondary antibody for 90 min at 37°C.

Immunohistochemical scoring

The expression of SFRP2 was estimated in an outcome-blinded model by two independent pathologists on a compound microscope. Briefly, the images were scored according to the staining depth: score 3: dark brown; score 2: brown madder; score 1: faint yellow; score 0: negative staining. Then the images were scored according to the number of positive cells in the total tumor cells, score 4: ≥76%; score 3: 51%-75%; score 2: 11%-50%; score 1: 1%-10%. The score of the same slide were summed to produce a final score (Final score = staining depth score × the number of positive cells score): strong positive (+++): ≥ score 5; middle positive (++): score 4; weakly positive (+): sore 3; negative (-): < score 3.

DNA isolation

DNA was extracted from Formalin-fixed, paraffin-embedded cancer tissues and their adjacent non-cancer specimens using the QIAamp DNA Formalin-fixed, paraffin-embedded kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was dissolved and stored at -20°C until use.

MSP

The methylation status of SFRP2 promoters in the bisulfite-modified DNA was determined by MSP. 2 μg DNA was bisulphite-treated using the Qiagen Epitect Fast Bisulfite Conversion kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The primer pairs were designed to discriminate between methylated and unmethylated alleles. Each primer pair mapped to nine cytosine-phosphate-guanine dinucleotide (CpG) sites in order to specifi-

Table 1. Primer sequences used in this study

Methylation-specific PCR	Sequence (5' → 3;)	T_A (°C)	bp
SFRP2 unmethylated	Forward: TTTTGGGTTGGAGTTTTTTGGAGTTGTGT	58	145
	Reverse: AACCCACTCTCTTCACTAAATACAACTCA		
SFRP2 methylated	Forward: GGGTCGGAGTTTTTCGGAGTTGCGC	58	138
	Reverse: CCGCTCTCTTCGCTAAATACGACTCG		

T_A, annealing temperature; bp, product size (base pairs).

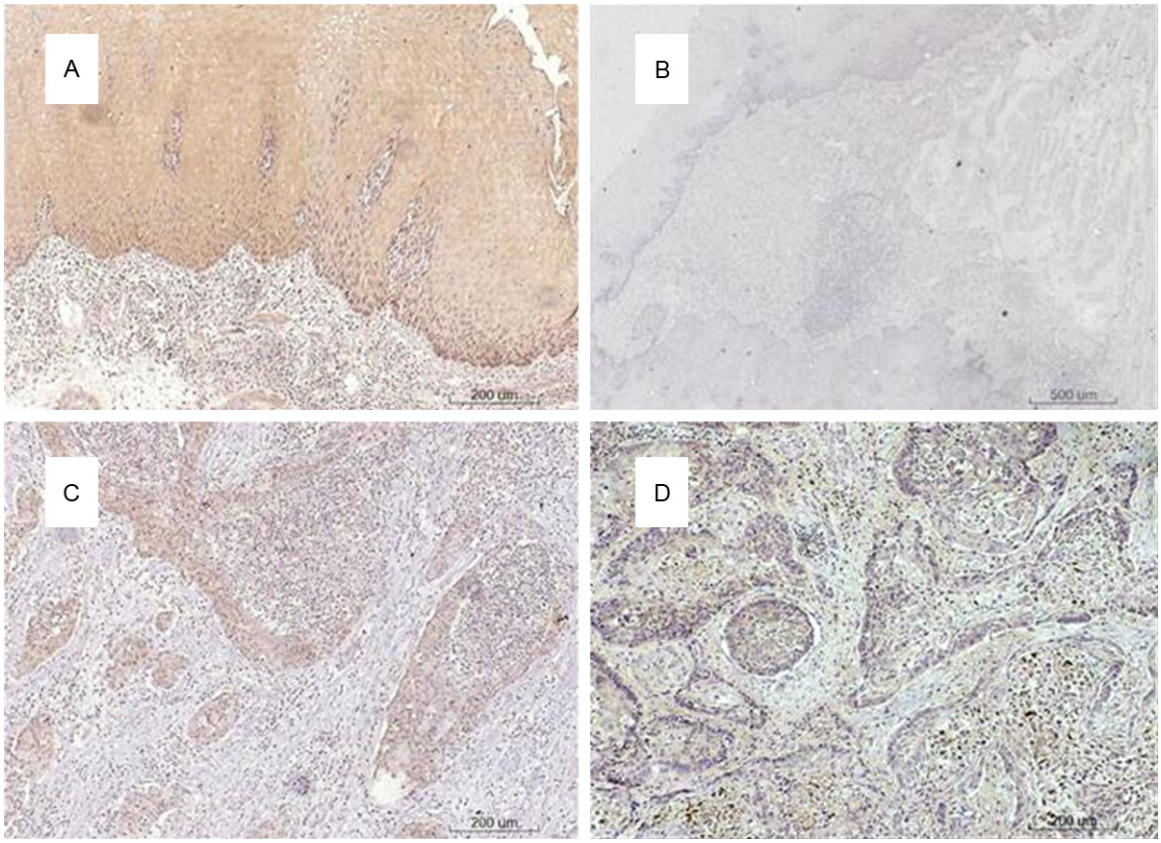

Figure 1. SFRP2 protein expression in ESCC tissues and normal controls. A. SFRP2 positive-expression in adjacent normal tissues (*10); B. SFRP2 negative-expression in adjacent normal tissues (*4); C. SFRP2 positive-expression in ESCC (*10); D. SFRP2 negative-expression in ESCC.

cally discriminate between methylated and non-methylated DNA. Further 11 non-CpG cytosines within the primer pair specific for methylated DNA and 13 non-CpG cytosines within the primer pair specific for non-methylated DNA guaranteed unequivocal amplification of bisulfite-converted DNA. All the primers were synthesized by Shanghai Biosune Biotechnology Company. The primer sequences used are shown in **Table 1**. The MSP conditions were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 40 s and a final extension at 72°C for 5 min. H_2O served as controls for the methylation and unmethylated promoter sequence, respectively. Amplification products were visualized on 3% low range ultra agarose gel containing ethidium bromide and illuminated under ultraviolet (UV) light.

Follow-up and survival analysis

Patients were followed up. The overall survival period was defined as the duration from the postoperative time point to death time point. The follow-up deadline was June 30, 2016. The median follow-up time was 53 months (range 8-84). Based on the follow-up data, the survival curves were made. The relations of SFRP2

Table 2. Correlation between the SFRP2 methylation gene promoter methylation status, SFRP2 protein expression level and clinicopathological parameters in ESCC

	ESCC					ESCC				
Characters	No	Low	High	χ^2	P	No	M	U	χ^2	P
Age, years										
<60	36	25	11	0.152	0.436	36	31	5	0.644	0.589
>60	64	42	22			64	51	13		
Gender										
Male	74	51	23	0.474	0.324	74	61	13	0.036	0.53
Female	26	16	10			26	21	5		
Nation										
Han	50	35	15	0.407	0.335	50	37	13	4.336	0.066
Hazakh	50	32	18			50	45	5		
Location										
Upper	3	2	1	0.623	0.732	3	2	1	0.739	0.691
Middle	46	29	17			46	37	9		
Lower	51	36	15			51	43	8		
Tumor Size										
<3	35	19	16	3.937	0.040	35	24	11	6.579	0.014
>3	65	48	17			65	58	7		
AJCC Stage										
T0	10	5	5	1.854	0.603	10	5	5	9.337	0.025
T1	33	22	11			33	26	7		
T2	30	22	8			30	27	3		
T3	27	18	9			27	24	3		
Infiltration										
Muscular	39	23	16	1.863	0.126	39	27	12	7.063	0.015
Mucous	61	44	17			69	55	6		
Lymph										
No	56	34	22	2.274	0.097	56	42	14	4.225	0.065
Yes	44	33	11			44	40	4		

χ^2: chi-squared test result; P: statistically significant (P<0.05); SFRP2: secreted frizzled-related protein 2; M: methylated; U: unmethylated; low, high: the expression of SFRP2 level.

expression with age, sex and lymph node metastasis were analyzed.

Statistical analysis

The SPSS 19.0 software (SPSS, Inc., Chicago, IL, United States) was used for all the statistical analysis. The continuous variables were expressed as means ± SEM. χ^2 test was used to assess the statistical significance of the correlations between SFRP2 expression and the different clinicopathological parameters respectively. Meanwhile, assess the association between the methylation gene and the different clinicopathological parameters. The patients were routinely followed-up clinically Overall survival (OS) was defined as time between date of surgery and date of death or the date of last follow-up. We used the Kaplan-Meier method and curves to calculate overall survival. Overall survival was defined as the time from the date of surgical resection to the date of death. Differences were indicated statistically significant when P was less than 0.05.

Results

Patient clinical characteristics

A total of 100 ESCC patients consisted of 50 Hans and 50 Kazakhs. Their mean age ± standard deviation were 64.72 ± 13.71. The mean age ± standard deviation of Hans with ESCC was 63.00 ± 13.71 years. While, that of the

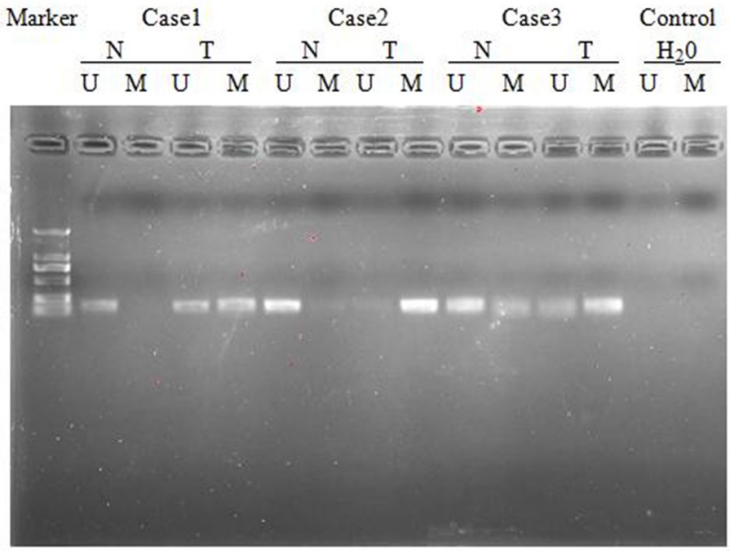

Figure 2. Representative results showing the SFRP2 promoter methylation status identified by MSP. MSP: methylation-specific polymerase chain reaction; M: methylated; U: unmethylated; T: ESCC tissues; N: corresponding normal tumor-adjacent tissues. control: blank control group.

Kazakhs was 65.26 ± 5.83 years, which was larger than the mean age of Hans. The ratio of male to female patients was 74:26. In addition, the rate of lymph node metastasis in the Kazakhs (21%) was slightly lower than the rate of lymph node metastasis in the Hans (23%). The 100 patients were classified according to AJCC as follow: pathological stage T0, 10 (10%); pathological stage T1, 33 (33%); pathological stage T2, 30 (30%); pathological stage T3, 27 (27%).

SFRP2 protein expression in ESCC tissues and normal controls

The positive immunostaining rate for SFRP2 in ESCC tissue was strongly lower than in adjacent normal tissues (33/100, 33% vs. 67/100, 67%) (**Figure 1**). The difference was significant ($P<0.01$). In addition, the SFRP2 expression in the tumors in Kazakhs was (17/50; 34%), which was slightly higher than that in the tumors in Hans (16/50; 32%). The statistic intimated that the expression status of SFRP2 was closely associated with the tumor size ($P = 0.04$), while, no association was found for SFRP2 protein expression and age, gender, nation, tumor location, tumor size, AJCC stage, infiltration degree and lymph node metastasis in ESCC. The results are listed in **Table 2**.

The promoter methylation of SFRP2 in ESCC and normal controls

To determine whether the DNA methylation status of SFRP2 gene in Formalin-fixed, paraffin-embedded cancer tissues had diagnostic value for ESCC, MSP analysis was used to investigate the frequency of DNA methylation of the gene in 100 ESCC patients. The methylation of the SFRP2 promoter was detected in 82 tumor samples (82%). However, in the corresponding normal tumor-adjacent tissues, the SFRP2 promoter was methylated in only 18 cases (18%). The frequency of SFRP2 promoter methylation in ESCC tissues was significantly higher than that in the adjacent tissues ($\chi^2 = 4.15$; $P = 0.052$). In addition, the SFRP2 gene methylation in the tumors in Kazakhs was (43/50; 86%), which was slightly higher than that in the tumors in Hans (39/50; 78%). Furthermore, the association between the SFRP2 methylation status and clinicopathological parameters were analyzed. The results were listed in **Table 2**. As indicated, methylation of the SFRP2 gene was significantly associated with, tumor size, AJCC stage and infiltration degree. However, there was no other correlation between the SFRP2v promoter methylation status and age gender nation tumor location lymph node metastasis. The agarose gel electrophoresis results of the MSP for the SFRP2 gene are shown in **Figure 2**.

SFRP2 protein expression and SFRP2 methylation status in ESCC patients

In our study, all the 82 cases with SFRP2 promoter methylation-positive ESCC tissues (82%), showed almost all lack of immunoreactivity for SFRP2, similar results were observed for the matched adjacent normal tissue. Interestingly, of the 18 cases with SFRP2 promoter methylation-negative tissues, 15 cases showed positive immunoreactivity for SFRP2 (15/18, 83%). There was a significant correlation between SFRP2 promoter hypermethylation and SFRP2 protein expression results ($\chi^2 = 25.153$, $P< 0.01$).

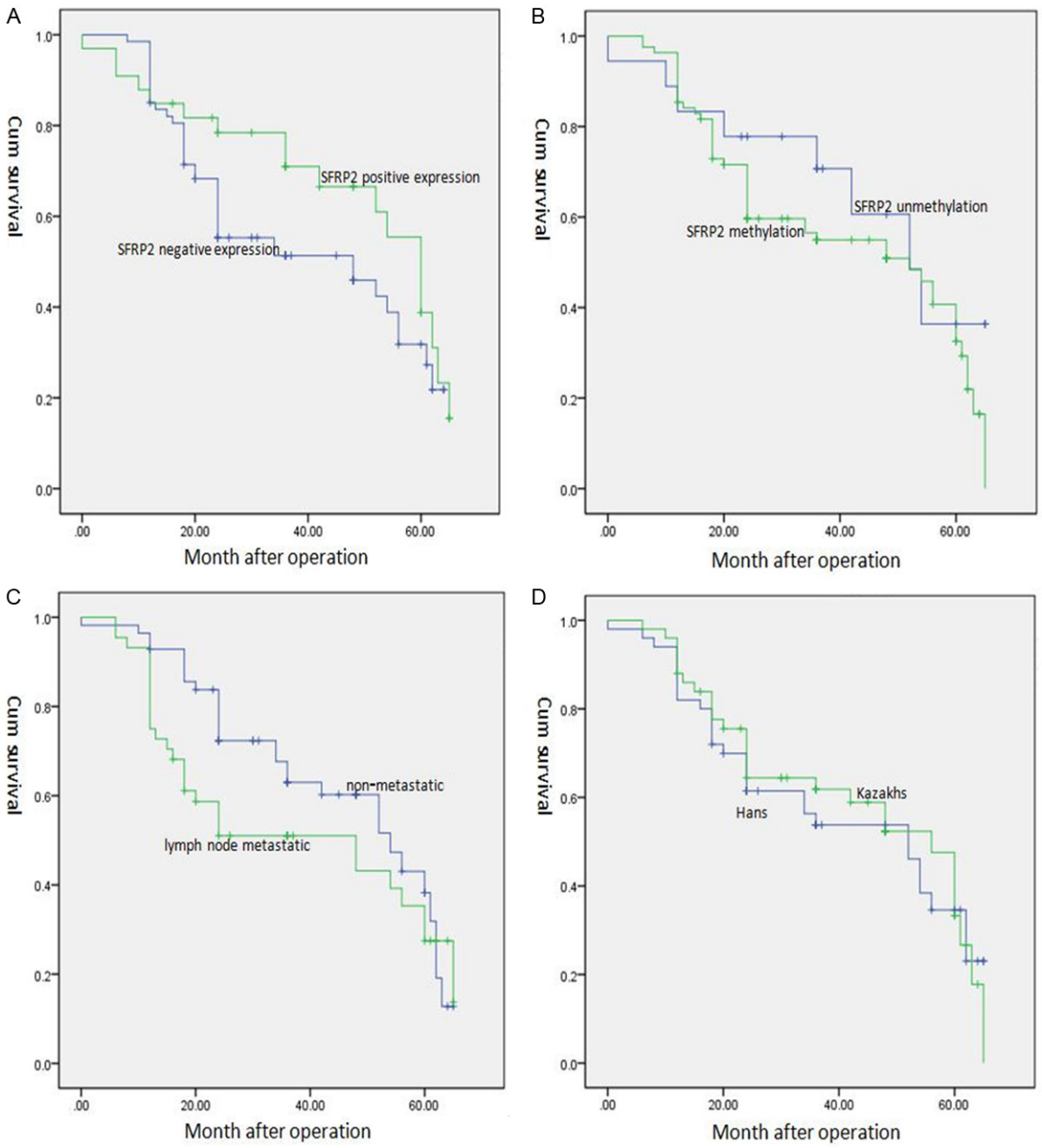

Figure 3. Kaplan-Meier survival curves for patients. A. SFRP2-positive expression with SFRP2-negative expression; B. Methylation of SFRP2 with unmethylation of SFRP2; C. Lymph node metastatic and non-metastatic; D. Hans and Kazakhs.

Prognostic significance of SFRP2 protein expression and SFRP2 promoter methylation status

100 follow-up patients during follow up periods of 1-65 months (median, 31 months) were analyzed by Survival dates. The overall survival rate of ESCC patients with SFRP2-positive expression was higher than the patients with SFRP2-negative expression, but not statistically significant (P>0.05) (**Figure 3A**); The 5-year survival rates for the patients was 12%. The Kaplan-Meier survival curves showed that the patients with methylation of SFRP2 had a poorer prognosis than those with unmethylation of SFRP2, but not statistically significant (P>0.05) (**Figure 3B**). Furthermore, there were no significant differences between lymph node metastatic group and non-metastatic group (χ^2 = 1.528, P = 0.216) (**Figure 3C**), Hans and Kazakhs (χ^2 =

0.023, P = 0.880) (**Figure 3D**), male patients and female patients (χ^2 = 0.034, P = 0.854), between ≥60 years old patients and <60 years old patients (χ^2 = 1.262, P = 0.261). (Figures were not shown).

Discussion

Esophageal cancer is the ninth most common cancer and the sixth leading cause of cancer-related mortality throughout the world [12-14]. Adenocarcinoma and squamous cell carcinoma are the principal histological types of esophageal cancer [15]. Esophageal cancer is often diagnosed at a very advanced stage and approximately half of all patients present with unresectable, locally advanced, or metastatic disease [16] Although esophageal adenocarcinoma is the most rapidly increasing cancer in Western countries, esophageal squamous cell carcinoma (ESCC) is still the dominant histological type around the world, which accounts for >95% of esophageal cancers in the People's Republic of China [17-19]. The mean 5-year survival rate for ESCC was estimated to be less than 30% [20]. Because of its extremely aggressive nature and poor survival rate, advanced ESCC is one of the least studied and deadliest cancers worldwide [21]. Thus, the key role involved in the development and progression of the disease must be investigated. SFRP2 may become a new underlying biology marker in the diagnosis and treatment of ESCC.

The SFRPs are a group of negative modulators of the Wnt signaling pathway, of which five members (SFRP1-5) have been identified to date. SFRP2 has been identified as a significant member of the SFRPs, which is associated with multiple tumor types [22, 23]. Silencing of SFRP2 protein expression is noted in the development of ESCC through promoter aberrant methylation. Probably, it may be a factor in ESCC progression by loss of its tumor-suppressive function. Recent Hao's study have demonstrated that SFRP2 was silenced in seven ESCC cell lines, but not in an immortalized esophageal epithelial cell line [24]. Similarly, in our analysis, we confirmed that the expression of SFRP2 was 2.4-fold lower in ESCC tissues than in paired noncancerous tissue samples, implying that down-regulation of SFRP2 may be involved in the carcinogenesis of ESCC. In addition, in our current study, we found that the expression of SFRP2 in Kazakhs was slight higher in Hans. Meanwhile, the methylation rate of SFRP2 was higher in Kazakh tissues than in Hans.

The silencing of expression was linked closely to promoter methylation, as also confirmed by Ma [25], they suggesting that promoter methylation is the principal regulatory mechanism of SFRP2 inactivation in ESCC. In our study, we investigated the expression levels of SFRP2 in ESCC specimens. We found that SFRP2 protein was significantly decreased, and this down-regulation of SFRP2 protein may result from the several possible reasons, including SFRP2 gene promoter methylation. Hence, we analyzed promoter methylation of SFRP2 in patients' specimens, and the results confirmed our hypothesis. The SFRP2 gene methylation was significantly increased in ESCC. Furthermore, MSP analysis of the SFRP2 gene promoter revealed a higher prevalence of CpG methylation in ESCC tissues than in adjacent non-tumorous tissues, suggesting that aberrant methylation of the SFRP2 promoter region is not a cell line-specific event but is frequent in ESCC carcinogenesis.

Nevertheless, there are some limitations in this study. The MSP method is a type of qualitative method, rather than quantitative method (such as Q-MSP), and could not provide exact values, only the number with methylation or not, leading to statistical deviation. Thus, we need to expand our sample size or use the Q-MSP combined pyrosequencing method to validate the results in the future.

Although a variety of molecular alterations have been identified over the last two decades, sensitive and specific biomarkers for early diagnosis and accurate indicators for ESCC prognosis are currently unavailable. Therefore, identification of targets for early detection of ESCC is important to improve the prognosis of patients with this pernicious disease [26]. The key finding in this report lies in, SFRP2 protein level and methylation status were both elevated in Chinese Han and Kazakh ethnic patients' specimens after surgery. However, whether SFRP2 expression is increased in precursor lesions of ESCC and other ESCC patients of different ethnicities needs further exploration.

Finally, in our study, we did not investigate that patients survival was correlated with the ex-

pressions and methylation status of SFRP2. However, we found little correlation with lymph node metastasis and survival. The limitations of this study were high loss of follow-up and a small sample size. Therefore, the relationship between the prognoses with the Wnt canonical signaling pathway may be investigated by further study with a large sample size.

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Disclosure of conflict of interest

None.

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