Int J Clin Exp Pathol 2016;9(6):6597-6609
www.ijcep.com /ISSN:1936-2625/IJCEP0023179

Original Article

Association between hypermethylation of the *SFRP1* promoter and cervical cancer in Han and Uyghur patients from Xinjiang

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Received January 3, 2016; Accepted March 17, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Purpose: DNA methylation is an early and frequent molecular change in the progression from precancerous lesions to invasive cancer. Silencing of the secreted frizzled-related protein 1 (*SFRP1*) gene through hypermethylation may be an important factor in the development of cervical cancer because *SFRP1* functions as a negative regulator of Wnt signaling that is commonly activated in human cancers. Methods: The methylation of 23 CpG sites in the *SFRP1* promoter was detected by MALDI-TOF mass spectrometry. In addition, polymerase chain reaction (PCR) was performed to detect infections with human papillomavirus 16 (HPV16) and determine the correlation between *SFRP1* methylation and HPV infection. Results: Methylation of the *SFRP1* CpG 2.3, 4, and 17 sites in cervical cancer samples from Uyghur patients was significantly higher than in samples from cervical intraepithelial neoplasia types 2 and 3 (CIN 2/3) (P = 0.003, 0.006, 0.006, respectively). The mean methylation levels of CpG 1, 7, 9.10, 12.13, 14.15, 18, and 25 were significantly increased in cervical cancer from Han patients compared with the CIN2/3 group (P = 0.003, 0.003, 0.001, 0.001, 0.001, 0.000, 0.001, respectively). HPV16 infection had no significant effect on the methylation of *SFRP1* in cervical cancer tissue from Uyghur or Han patients (P > 0.05). Conclusion: Our study demonstrated that hypermethylation of different CpG sites of the *SFRP1* gene, occurring during the evolution of CIN 2/3 to cervical cancer, was associated with cervical carcinogenesis in both Uyghur and Han patients, but that there were differences between these groups suggesting ethnic differences in genetic susceptibility to cervical cancer.

Keywords: *SFRP1*, cervical cancer, Uyghur, Han, methylation, HPV

Introduction

Cervical cancer is the third leading cause of cancer-related death in women worldwide. The American Cancer Society estimates that, in 2013 in the United States, 12,340 women will be diagnosed with malignant cervical cancer, and 4,030 women will die from this disease [1]. In China, as the most common malignant tumor of the female genital system, there are an estimated 140,000 new cases of, and 80,000 deaths attributed to, cervical cancer annually [2]. Moreover, the incidence of this disease in young Chinese women (≤ 30 years old) is increasing by 2% to 3% yearly. The incidence of cervical cancer in the Uyghur ethnic group from the Xinjiang region of China is much higher than that of the Han ethnic group. Although HPV infection is the most common risk factor for cervical cancer, environmental, genetic, and/or epigenetic alterations also play crucial roles in the progression from precancerous disease to invasive cancer [3-7].

The Wnt/β-catenin signaling pathway plays a key role in regulating embryonic development, as well as in cell proliferation, differentiation, and migration [8]. Recent reports provide evidence that the abnormal activation of the Wnt/β-catenin pathway can inhibit tumor cells apop-

tosis in several human cancers, including melanomas, breast cancer, colorectal cancer, and hepatocellular carcinoma [9-12]. When present Wnt ligands bind to the transmembrane receptor frizzled, and the signal is transduced to the cytoplasmic protein disheveled by phosphorylation. Through a series of molecular interactions, β-catenin then accumulates and enters the nucleus where it forms a complex with T-cell factor/lymphocyte-enhanced factor (TCF/LEF) and upregulates TCF/LEF-dependent transcription of target genes such as c-myc and cyclin D1 [13]. There is also functional evidence for the deregulation of the Wnt signaling pathway during high-risk human papillomavirus (HPV)-mediated transformation in vitro [14-16].

Secreted frizzled-related proteins (SFRPs), a family of five secreted glycoproteins including SFRP1, SFRP2, SFRP4, and SFRP5, are extracellular signaling molecules that antagonize the Wnt signaling pathway. In a previous study, it was demonstrated that *SFRP* genes are candidate tumor suppressor genes (*TSG*s) that are silenced in cervical carcinogenesis through promoter hypermethylation, especially *SFRP1* [17-19]. SFRP1 serves as a signaling ligand by binding Wnt proteins and frizzled receptors in the extracellular compartment [20]. SFRP1 modulates the Wnt pathway by keeping Wnt proteins from combining with the frizzled receptors, thereby preventing initiation of the signaling cascade [21, 22]. Ko et al. reported the absence of *SFRP1* expression in cervical cancer patients. Recently, additional studies showed that *SFRP1* is a candidate *TSG* that is frequently down-regulated through hypermethylation of its promoter in cervical carcinoma [17-19]. Therefore, the loss of *SFRP1* activity through promoter methylation, leading to overactivation of the Wnt signaling pathway, ultimately promotes tumorigenesis in varied human cancers, including cervical carcinoma [23-25].

Aberrant hypermethylation of CpG islands, which are CpG dinucleotide-rich areas located mainly in the promoter regions of many genes, serves as an alternative mechanism for inactivation of *TSG*s in cancers [26-28]. Such hypermethylation of gene promoters has been increasingly considered as an early event in cervical carcinogenesis [27-30]. Based on the observations described above, measurement of *SFRP1* gene inactivation by promoter methylation might be used as an epigenetic biomarker for cervical cancer. It is noteworthy that methylation of the *SFRP1* gene among Uyghur women has not been reported previously. In addition, we were interested in which CpG units in *SFRP1* are related to gene promoter methylation, and the difference between Han and Uyghur patients.

In the present study, we used the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to detect the methylation status of *SFRP1* among Uyghur and Han populations. We quantitatively evaluated the individual methylation of CpG units in regions 333 base pairs in length containing 23 CpG sites within 15 CpG units of the *SFRP1* promoter regions in a total of 301 patients The association between the extent of methylation of multiple CpG units of *SFRP1* promoters and cervical cancer was also investigated. In order to understand the correlation and variation between the methylation of the *SFRP1* gene promoter and infection with HPV16, the presence of HPV16 in samples from Uyghur and Han patients was determined by the polymerase chain reaction (PCR).

Materials and methods

Sample collection

Three hundred and one cervical tissues were randomly collected by multistage cluster sampling from Han and Uyghur patients diagnosed with histologically confirmed invasive cervical cancer (ICC) (58 cases of Han, 54 cases of Uyghur), cervical intraepithelial neoplasia types 2 and 3 (CIN2/3) (57 cases of Han and 36 cases of Uyghur), cervical intraepithelial neoplasia types 1 (CIN1) (36 cases of Han, 11 cases of Uyghur), and normal cervical tissues (28 cases of Han and 21 cases of Uyghur). Supplementary Table 1 summarizes the incidence of cervical cancer in the two ethnic groups. Patient ages ranged from 24 to 80 years, with a median age of 46 years. All patients were recruited between 2005 and 2009 from the First Affiliated Hospital of Shihezi University and the People's Hospital of the Kashgar Region. All samples were resected by surgery, fixed in 10% buffered formalin, rou-

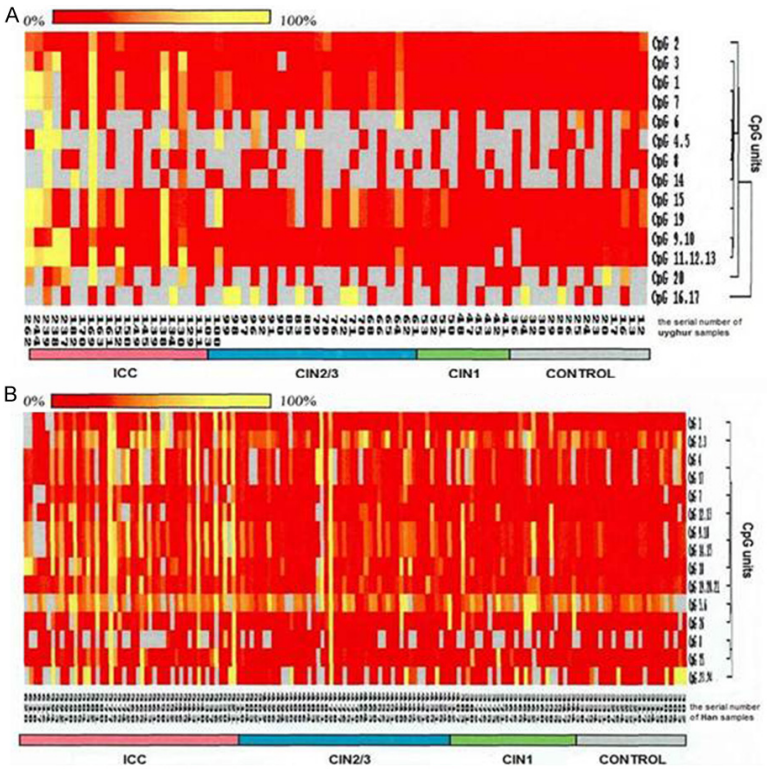

Figure 1. Hierarchical cluster analysis of the methylation of CpG units of the *SFRP1* promoter region in tissue samples from invasive cervical cancer, CIN2/3, CIN1, and normal groups. A. Uyghur samples. B. Han samples. Columns display the clustering of CpG units, which is a single CpG site or a combination of CpG sites. Each row represents a sample. The methylation intensity of each *SFRP1* CpG unit in each sample varies from red to yellow, which represents high (100) to low (0) expression. Gray represents technically inadequate or missing data.

tinely processed, and embedded in paraffin. The diagnoses of all tissues, using hematoxylin-eosin and immunohistochemistry stained slides from each case, were confirmed by two experienced pathologists according to the WHO Pathology & Genetics Tumours of the Breast and Female Genital Organs (seventh edition).

This study was approved by the Research Ethics Committee of Shihezi University School of Medicine, P. R. China. Written informed consent was obtained from all patients. All samples were handled and anonymized according to approved ethical and legal standards.

Isolation of genomic DNA and bisulfite conversion

DNA was extracted from 15 tissue slices of 10 μm thickness by proteinase K digestion and a tissue DNA extraction kit (Qiagen Inc., Valencia, CA, USA) based on the manufacturer's instructions. The genomic DNA was stored at -20°C until used as a template for each PCR reaction. According to the manufacturer's protocol, genomic DNA was treated with bisulfite though an EZ DNA Methylation KitTM (Zymo Research, Orange Country, CA, USA). This treatment combines bisulfite conversion and DNA clean-up. DNA concentrations and quality were assessed with a ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

PCR analysis

As an internal control, all purified genomic DNA samples were validated by PCR with a human β-globin primer (forward: 5'-CAACTTCATCCACGTTCACC-3'; reverse: 5'-GAAGAGCCAAGGACAGGTAC-3'), to determine that the quality and quantity of DNA were suitable for detecting the presence of HPV16 and the *SFRP1* methylation profile (Supplementary Table 2). PCR analysis of HPV16 was performed using a modified HPV 16 primer set (forward: 5'-GACCCAGAAAGTTACCACAG-3'; reverse: 5'-CACAACGGTTTGTTGTATTG-3').

MassARRAY analysis

The CpG island sequences were identified by the Human Genome Browser Gateway (http://genome.ucsc.edu/). The analyzed region and the CpG sites of the SFRP1 promoter are shown in **Figure 1**. Primer sets for the methylation analysis of the *SFRP1* promoter region were designed using EpiDesigner software (http://epidesigner.com; **Table 2**). The methylation of *SFRP1* DNA was quantitatively analyzed by the MassARRAY platform (SEQUENOM) as described previously [31]. The PCR mixture (3 μl) contained 10 ng of bisulfite-treated DNA, 25 mM dNTP, 0.2 U of Hot Start TaqDNA poly-

Table 1. Correlation between SFRP1 promoter methylation and four groups of cervical disease in Uyghur and Han patients

CpG units	Ethnicity	The average extent of methylation ± Standard deviation				P_1	P_2	P_3	P_4	P_5	P_6
		Normal	CIN1	CIN2/3	ICC						
CpG1	U	0.166 ± 0.061	0.099 ± 0.010	0.197 ± 0.053	0.187 ± 0.033	0.377	0.420	0.446	0.771	0.049*	0.004*
	H	0.094 ± 0.008	0.136 ± 0.020	0.130 ± 0.019	0.233 ± 0.036						
CpG2.3	U	0.231 ± 0.031	0.186 ± 0.021	0.195 ± 0.030	0.270 ± 0.025	0.162	0.240	0.002*	0.014*	0.022*	0.058
	H	0.239 ± 0.018	0.287 ± 0.027	0.264 ± 0.019	0.346 ± 0.033						
CpG4	U	0.137 ± 0.011	0.116 ± 0.024	0.105 ± 0.011	0.188 ± 0.026	0.162	0.240	0.002*	0.014*	0.022*	0.058
	H	0.177 ± 0.021	0.163 ± 0.020	0.212 ± 0.027	0.284 ± 0.033						
CpG5.6	U	0.278 ± 0.020	0.283 ± 0.041	0.485 ± 0.077	0.441 ± 0.077	0.127	0.161	0.045*	0.683	0.075	0.054
	H	0.311 ± 0.012	0.397 ± 0.041	0.361 ± 0.032	0.414 ± 0.033						
CpG7	U	0.099 ± 0.006	0.107 ± 0.006	0.089 ± 0.007	0.114 ± 0.011	0.038*	0.418	0.005*	0.000*	0.455	0.003*
	H	0.119 ± 0.007	0.121 ± 0.010	0.143 ± 0.017	0.182 ± 0.014						
CpG8	U	0.043 ± 0.013	0.054 ± 0.024	0.043 ± 0.013	0.130 ± 0.035	0.589	0.888	0.818	0.375	0.045*	0.063
	H	0.042 ± 0.008	0.056 ± 0.010	0.056 ± 0.022	0.151 ± 0.048						
CpG9.10	U	0.153 ± 0.010	0.168 ± 0.013	0.144 ± 0.012	0.200 ± 0.022	0.001*	0.013*	0.000*	0.000*	0.327	0.001*
	H	0.203 ± 0.008	0.240 ± 0.023	0.233 ± 0.016	0.344 ± 0.029						
CpG12.13	U	0.088 ± 0.012	0.084 ± 0.012	0.077 ± 0.010	0.147 ± 0.026	0.452	0.898	0.900	0.117	0.250	0.003*
	H	0.077 ± 0.008	0.135 ± 0.028	0.112 ± 0.023	0.240 ± 0.037						
CpG14.15	U	0.153 ± 0.010	0.168± 0.013	0.144 ± 0.012	0.200 ± 0.022	0.001*	0.013*	0.000*	0.000*	0.327	0.001*
	H	0.203 ± 0.008	0.240 ± 0.023	0.233 ± 0.016	0.344 ± 0.029						
CpG17	U	0.137 ± 0.011	0.116 ± 0.024	0.106 ± 0.011	0.188 ± 0.024	0.162	0.240	0.002*	0.014*	0.022*	0.058
	H	0.177 ± 0.021	0.163 ± 0.020	0.212 ± 0.027	0.284 ± 0.033						
CpG18	U	0.174 ±0.012	0.154 ± 0.013	0.195 ± 0.013	0.257 ± 0.031	0.047*	0.784	0.009*	0.287	0.088	0.000*
	H	0.138 ± 0.007	0.178 ± 0.020	0.203 ± 0.029	0.334 ± 0.036						
CpG19.20.21	U	0.089 ± 0.006	0.091 ± 0.008	0.114 ± 0.008	0.132 ± 0.010	0.000*	0.000*	0.000*	0.000*	0.009*	0.621
	H	0.182 ±0.010	0.202 ± 0.0154	0.219 ± 0.018	0.240 ± 0.020						
CpG23.24	U	0.188 ± 0.044	0.308 ± 0.107	0.338 ± 0.073	0.369 ± 0.069	0.401	0.246	0.002*	0.105	0.154	0.132
	H	0.262 ± 0.059	0.248 ± 0.064	0.181 ± 0.029	0.271 ± 0.038						
CpG25	U	0.109 ± 0.011	0.113 ± 0.010	0.102 ± 0.010	0.173 ± 0.024	0.549	0.373	0.736	0.698	0.088	0.005*
	H	0.120 ± 0.011	0.137 ± 0.031	0.121 ± 0.020	0.217 ± 0.031						
CpG26	U	0.084 ± 0.0494	0.095 ± 0.039	0.137 ± 0.047	0.211 ± 0.044	0.292	0.315	0.700	0.246	0.026*	0.453
	H	0.101 ± 0.008	0.214 ± 0.056	0.143 ± 0.026	0.171 ± 0.027						

Note: Normal, normal cervical epithelium; CIN1, cervical intraepithelial neoplasia 1; CIN2/3, cervical intraepithelial neoplasia 2 and 3; ICC, invasive cervical cancer; U, Uyghur; H, Han; P1, P2, P3, P4 values for the correlation of the methylation level of the same CpG units between the Uyghur and Han patients with normal cervical epithelium, CIN1, CIN2/3 and cervical cancer, respectively (Mann-Whitney U-test); P5 and P6 are the correlation of the methylation level of the same CpG unit between normal cervical epithelium, CIN1, CIN2/3 and cervical cancer groups in Uyghur and Han patients, respectively (Kruskal-Wallis H-test); *$P < 0.05$.

Table 2. Comparison of the extent of methylation of each CpG unit between two cervical groups in Uyghur and Han patients

CpG units	Ethnicity	a'					
		Normal vs CIN1	Normal vs CIN2/3	Normal vs cancer	CIN1 vs CIN2/3	CIN1 vs cancer	CIN2/3 vs cancer
CpG1	U	0.972	0.727	0.055	0.477	0.022	0.042
	H	0.173	0.669	0.001*	0.448	0.059	0.003*
CpG2.3	U	0.411	0.042	0.264	0.325	0.088	0.003*
	H	0.137	0.742	0.132	0.261	0.625	0.135
CpG4	U	0.203	0.08	0.256	0.927	0.083	0.006*
	H	0.531	0.746	0.054	0.364	0.015	0.075
CpG5.6	U	0.932	0.022	0.104	0.136	0.237	0.252
	H	0.219	0.623	0.032	0.169	0.55	0.017
CpG7	U	0.275	0.683	0.401	0.26	0.932	0.176
	H	0.961	0.766	0.002*	0.611	0.005*	0.003*
CpG8	U	0.479	0.986	0.024	0.626	0.24	0.012
	H	0.329	0.282	0.198	0.059	0.787	0.02
CpG9.10	U	0.277	0.991	0.186	0.457	0.701	0.111
	H	0.282	0.366	0.000*	0.996	0.012	0.001*
CpG12.13	U	0.979	0.457	0.289	0.533	0.464	0.048
	H	0.591	0.748	0.002*	0.431	0.035	0.001*
CpG14.15	U	0.277	0.991	0.186	0.457	0.701	0.111
	H	0.282	0.366	0.000*	0.996	0.012	0.001*
CpG17	U	0.203	0.08	0.256	0.927	0.083	0.006*
	H	0.531	0.746	0.054	0.364	0.015	0.075
CpG18	U	0.589	0.248	0.088	0.067	0.053	0.271
	H	0.257	0.755	0.000*	0.517	0.002*	0.000*
CpG19.20.21	U	0.876	0.058	0.003*	0.119	0.023	0.281
	H	0.603	0.484	0.196	0.912	0.395	0.526
CpG23.24	U	0.459	0.118	0.021	0.707	0.394	0.576
	H	0.64	0.39	0.288	0.949	0.181	0.014
CpG25	U	0.587	0.807	0.06	0.796	0.25	0.022
	H	0.275	0.222	0.08	0.784	0.011	0.001*
CpG26	U	0.925	0.55	0.011	0.56	0.11	0.024
	H	0.401	0.684	0.38	0.196	0.924	0.207

Note: a' values were calculated by the correction formula: a' = a/N, N = Cn2 = n(n-1)/2, a' = 0.0083; *Indicates a' < 0.0083.

merase (Sequenom, San Diego, CA, USA), and a 1 μM primers mix. The cycles included preheating at 94°C for 4 min, followed by incubation for 45 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 60 s. Samples were then incubated at 72°C for 3 min.

Two microliters of a shrimp alkaline phosphatase (SAP) mix containing 1.7 μl of H_2O and 0.3 μl of SAP (Sequenom) were added to digest redundant dNTPs with the following program: 37°C for 20 min, 85°C for 5 min, and 4°C thereafter. After the SAP treatment, unincorporated dNTPs were dephosphorylated by adding 2 ml of a premix containing 0.3 U of SAP (Sequenom). The reaction mixture was incubated at 37°C for 40 min. The SAP was then heat-inactivated for 5 min at 85°C and maintained at 4°C. Five microliters of T Cleavage Transcription/RNase Cocktail containing 0.89 μl of 5 × T7 polymerase buffer, 0.24 μl of T cleavage mix, 3.14 mM dithiothreitol, 22 U of T7 RNA and DNA polymerase, 0.09 mg/ml of RNase A, and 2 μl of the product of the PCR/SAP reactions were mixed and incubated at 37°C for 3 h of in vitro transcription and RNase

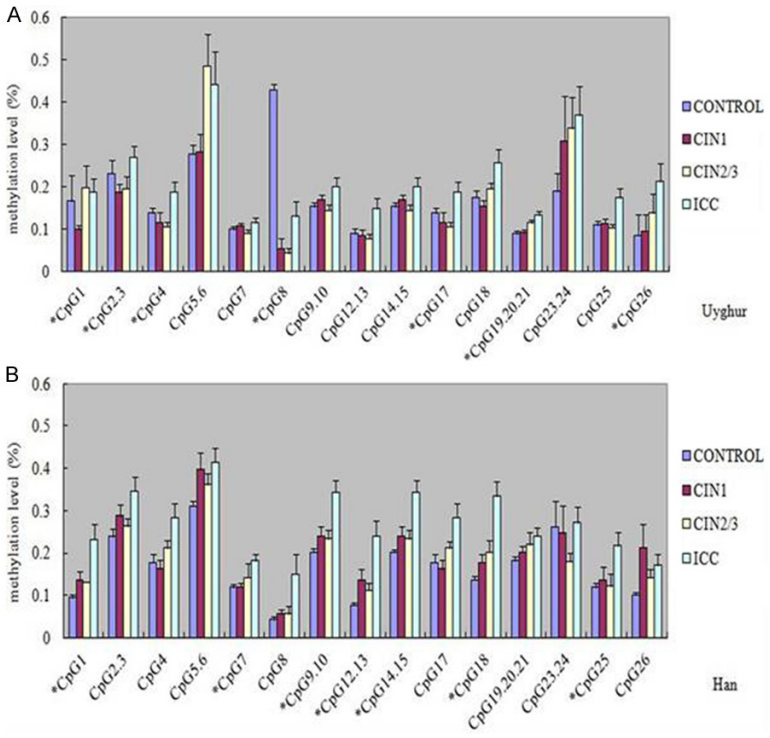

Figure 2. Evaluation of methylation of the *SFRP1* promoter. Mean methylation levels of 15 informative CpG units in the *SFRP1* promoter among 56tn-normal, CIN2, CIN2/3, and ICC from A. Uyghur, and B. Han patients. *P < 0.05 (Kruskal-Wallis H-test). Error bars represent standard deviation.

A digestion. Fifteen nanoliters of the cleavage reaction were then robotically dispensed (by a nanodispenser) onto silicon chips preloaded with a matrix (SpectroCHIP; Sequenom). Mass spectra were collected by a MassARRAY Compact MALDI-TOF (Sequenom). Methylation data of individual units (one to three CpG sites per unit) were generated using EpiTyper v1.0.5 software (Sequenom). All experiments were performed in triplicate. Non-applicable readings and their corresponding site were eliminated by calculation. The methylation level was expressed as the percentage of methylated cytosines relative to the total number of methylated and unmethylated cytosines.

Statistical methods

Statistical analyses were performed with the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). The correlation between each CpG methylation site of *SFRP1* and the two ethnic groups studied was evaluated by a nonparametric test (Mann-Whitney U-test; two-sided). A Kruskal-Wallis H-test (two-sided) was used to compare the *SFRP1* methylation levels of every CpG site among ICC, CIN2/3, CIN1, and normal cervical tissues between Uyghur and Han patients. All values are presented as means ± standard deviation. Significance was set at a P value of < 0.05. Statistical comparisons between any two groups from ICC, CIN1, CIN2/3, and normal were performed with the correction formula: $a' = a/N$, $N = C_n^2 = n(n-1)/2$, $a' = 0.0083$.

Results

DNA methylation of the SFRP1 gene in cervical cancer, CIN2/3, CIN1, and normal groups from Han and Uyghur patients

The quantitative analysis of methylated DNA by Sequenom MassARRAY, a mass spectrometry method, is based on the detection of the methylation status of a single CpG unit at a target fragment (CpG island) and generates data indicating the ratio or frequency of the methylation events on a CpG unit in all DNA samples. This system was used to evaluate the methylation profile of *SFRP1* in the samples from Uyghur and Han patients. The amplicon detected in the promoter of *SFRP1* was 333 base pairs in length and included 23 CpG sites that can be divided into 15 CpG units.

The final dataset consisted of 15 CpG units (determined from 1,425 sites in 95 Uyghur samples and 2,250 sites in 150 Han samples). The methylation of individual CpG units in *SFRP1* is depicted in the cluster diagram (**Figure 1**). The incidence of methylation in Uyghur and Han women was 83% (1181/1425) and 91% (2045/2250), respectively. However, many CpG units were methylated to a very low degree. Specifically, only 5.2% and 7.6% of the *SFRP1* CpG units had mean methylation levels greater than 50% in Uyghur and Han people, respectively. This methylation was mostly in CIN2/3 and cervical squamous cell carcinoma tissues. These results indicate that the methyl-

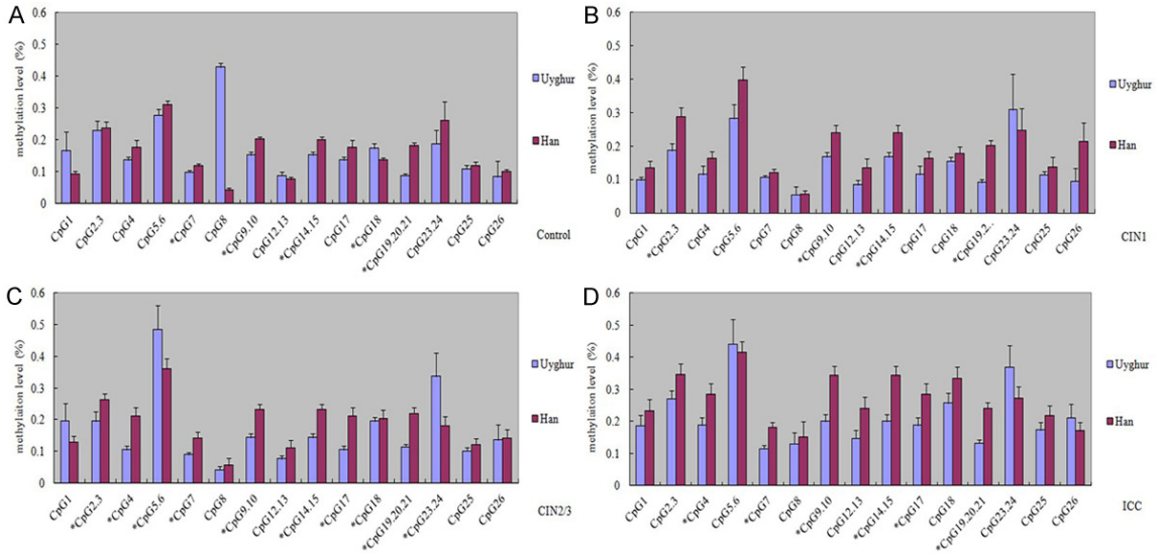

Figure 3. Association between the level of *SFRP1* methylation and two ethnic groups in four tissue samples. A. Normal, normal cervical epithelium samples. B. CIN1, cervical intraepithelial neoplasia 1. C. CIN2/3, cervical intraepithelial neoplasia 2 and 3. D. ICC, invasive cervical cancer. The mean methylation value for most CpG in cervical samples from Han patients is higher than that from Uyghur patients. *$P < 0.05$ (Mann-Whitney U-test). Error bars represent standard deviation.

ation levels of the *SFRP1* promoter were greater in Han than in Uyghur people. Overall, the methylation status of tumor tissues was obviously different from that observed in three noncancerous tissues.

DNA methylation of the SFRP1 gene in four cervical groups of Uyghur patients

A cluster analysis was used to analyze the distribution of the methylation of 15 CpG units of the *SFRP1* gene amplicon in all cervical samples from Uyghur patients. We found that the methylation level of cervical cancer DNA was higher than that in the normal cervical tissues, CIN1, and CIN2/3 groups (**Figure 1**). Specifically, based on the Kruskal-Wallis H test, single CpG site methylation levels from Uyghur cervical cancer, CIN2/3, CIN1, and normal groups were significantly different among CpG1, CpG2.3, CpG4, CpG8, CpG17, CpG-19.20.21, and CpG26 units ($P = 0.049$, 0.022, 0.022, 0.045, 0.022, 0.009, and 0.026, respectively). In addition, the extent of methylation of DNA from cervical cancer was generally higher than in other groups, and the average methylation of each CpG unit was distinct (**Table 1** and **Figure 2**).

Comparing the extent of methylation of each CpG unit between two cervical groups (normal vs. CIN1, normal vs. CIN2/3, normal vs. cancer, CIN1 vs. CIN2/3, CIN1 vs. cancer, and CIN2/3 vs. cancer), using the new inspection standard to judge the results, showed that CpG19.20.21 methylation between cervical cancer and the normal groups was significantly different ($a' = 0.003$). In addition, CpG2.3, CpG4, and CpG17 methylation in cervical cancer was higher than in the CIN2/3 groups ($a' = 0.003$, 0.006, 0.006, respectively) (**Table 2**).

DNA methylation of the SFRP1 gene in four cervical groups of Han patients

A cluster analysis was used to analyze the distribution of the methylation of 15 CpG units of the *SFRP1* gene amplicon in all cervical samples from Han patients. We found that the methylation level of cervical cancer DNA was higher than normal, CIN1, and CIN2/3 (**Figure 1**). Specifically, as shown in **Table 1** and **Figure 2**, methylation levels among CpG1, CpG7, CpG9.10, CpG12.13, CpG14.15, CpG18, and CpG25 sites were significantly different among the four Han groups ($P = 0.04$, 0.003, 0.001, 0.003, 0.001, 0.000, 0.005, respectively). In addition, the mean extent of DNA methylation was increased in the Han cervical squamous cell carcinomas group compared with the CIN2/3, CIN1, and normal groups (**Table 1** and **Figure 2**).

Table 3. The HPV16 infection in Han and Uyghur samples (%)

Han	Normal	CIN1	CIN2/3	Cancer	χ_1^2	P_1	χ_2^2	P_2
HPV +	0 (0)	1 (2.8)	30 (52.6)	41 (70.7)	22.775	0.00*	37.827	0.00*
HPV -	28 (100)	35 (97.2)	27 (47.4)	17 (29.3)				
Uyghur	Normal	CIN1	CIN2/3	Cancer	χ_1^2	P_1	χ_2^2	P_2
HPV +	1 (4.8)	3 (27.3)	15 (41.7)	40 (74.1)	38.075	0.00*	95.371	0.00*
HPV -	20 (95.2)	8 (72.7)	21 (58.3)	14 (25.9)				

Note: χ_1^2, P_1: CIN2/3 with normal; χ_2^2, P_2: cancer with normal; *: $P < 0.05$.

Comparing the extent of methylation of CpG units between two cervical groups, there was greater methylation of CpG7 (cancer vs normal, $P = 0.002$; cancer vs CIN1, $P = 0.005$, cancer vs. CIN2/3, $P = 0.003$) and CpG18 (cancer vs normal, $P < 0.001$; cancer vs CIN1, $P = 0.002$, cancer vs CIN2/3, $P < 0.001$) in cervical cancer than in the three other groups. In addition, the methylation of CpG1, CpG9.10, CpG12.13, and CpG14.15 units of cervical cancer was significantly higher than normal (a' = 0.001, 0.001, 0.002, 0.001 respectively) and CIN2/3 ($P = 0.003$, 0.001, 0.001, 0.001, respectively). However, there was no significant difference between cancer and CIN1. The differences between CIN2/3, CIN1, and normal were not significant ($a' > 0.0083$) (**Table 2**).

Methylation of the SFRP1 gene promoter region in Uyghur and Han populations

The methylation level of *SFRP1* was greater in the Han samples than in the Uyghur samples when comparing patients with normal cervical epithelium, CIN1, CIN2/3, and cervical cancer by the Mann-Whitney U-test (normal group: CpG7, CpG9.10, CpG14.15, CpG18, CpG19.20.21; CIN1 group: CpG2.3, CpG9.10, CpG14.15, CpG19.20.21; CIN2/3 group: CpG2.3, CpG4, CpG5.6, CpG7, CpG9.10, CpG14.15, CpG17, CpG18, CpG19.20.21, CpG23.24; cancer group: CpG4, CpG7, CpG9.10, CpG14.15, CpG17, CpG19.20.21; $P < 0.05$) (**Table 1** and **Figure 3**).

The association between the methylation of SFRP1 and HPV16 infection in cervical carcinoma

The prevalence of HPV16 infection in Han patients is shown in **Table 3**. Infections ranged from 53% in CIN2/3 and 70% in cancer, to 0% in normal (CIN2/3 vs normal: $\chi^2 = 22.775$, $P_1 = 0.00$; cancer vs normal: $\chi^2 = 37.827$, $P_2 = 0.00$; $P < 0.05$). Infection with HPV16 was more frequent in CIN-2/3 (42%) and cancer (74%) than normal (5%) among Uyghur patients (CIN2/3 vs normal: $\chi^2 = 38.075$, $P_1 = 0.00$; cancer vs. normal: $\chi^2 = 95.371$, $P_2 = 0.00$; $P < 0.05$). There were no correlations between the methylation of *SFRP1* and infection with HPV16 in Uyghur and Han patients with cervical carcinoma (**Table 4** and **Figure 4**).

Discussion

The development of cervical cancer can take decades. Thus, adequate detection and treatment of preneoplastic lesions might reduce the development of this invasive cancer [32, 33]. In malignant tissues, while most portions of the genome exhibit global hypomethylation, CpG islands within gene promoter regions undergo hypermethylation. DNA methylation patterns are altered in many human neoplasia and could be used to improve cancer diagnosis [34, 35]. Nevertheless, despite progress, there is still no reliable biomarker for diagnosing cervical cancer [6].

Wnt signaling pathways play important roles in the regulation of embryonic development, cell proliferation, and differentiation. The abnormal activation of Wnt can influence the transcription of multiple proto-oncogenes leading to tumor development. Previous studies have suggested that hypermethylation of the *SFRP1* gene occurs in liver cancer [36], breast cancer [23], squamous cell carcinoma of the head and neck [37], and oral squamous cell carcinoma [38]. Other researchers [39] found that the *SFRP1* gene was 58% methylated in cervical cancer, which was significantly higher than the 5% seen in normal controls. In Chung's research, methylation of the *SFRP1* gene in cervical cancer cell lines, high-grade intraepithelial lesions, low level intraepithelial lesions, and normal cervical cells was 33.9%, 8.2%, 2.2%, and 0%, respectively [18]. Although the specific molecular mechanism of cervical cancer development is not fully understood, a reasonable explanation may be that methylation of the *SFRP1* promoter causes the transcriptional inactivation and downregulation of *SFRP1*.

Table 4. Association between SFRP1 promoter methylation and infection with HPV16 in Uyghur and Han patients with cervical cancer

CpG units	(Uyghur) Average extent of methylation ± standard deviation		*P* value	(Han) Average extent of methylation ± standard deviation		*P* value
	HPV16 +	HPV16 -		HPV16 +	HPV16 -	
CpG1	0.177 ± 0.039	0.221 ± 0.058	0.266	0.242 ± 0.048	0.200 ± 0.046	0.690
CpG2.3	0.264 ± 0.025	0.286 ± 0.069	0.804	0.344 ± 0.037	0.354 ± 0.059	0.776
CpG4	0.179 ± 0.021	0.224 ± 0.073	0.660	0.286 ± 0.036	0.277 ± 0.070	0.728
CpG5.6	0.475 ± 0.096	0.340 ± 0.020	0.504	0.429 ± 0.045	0.367 ± 0.023	0.828
CpG7	0.110 ± 0.010	0.134 ± 0.046	0.857	0.187 ± 0.018	0.168 ± 0.023	0.438
CpG8	0.105 ± 0.030	0.263 ± 0.148	0.104	0.169 ± 0.069	0.109 ± 0.051	0.655
CpG9.10	0.196 ± 0.020	0.213 ± 0.071	0.360	0.359 ± 0.037	0.299 ± 0.037	0.302
CpG12.13	0.130 ± 0.022	0.200 ± 0.078	0.745	0.262 ± 0.051	0.178 ± 0.043	0.492
CpG14.15	0.196 ± 0.020	0.213 ± 0.071	0.360	0.359 ± 0.037	0.299 ± 0.037	0.302
CpG17	0.179 ± 0.021	0.224 ± 0.073	0.660	0.286 ± 0.036	0.277 ± 0.070	0.728
CpG18	0.247 ± 0.030	0.296 ± 0.098	0.843	0.327 ± 0.043	0.356 ± 0.073	0.857
CpG19.20.21	0.133 ± 0.011	0.129 ± 0.034	0.541	0.232 ± 0.024	0.267 ± 0.039	0.531
CpG23.24	0.365 ± 0.075	0.400 ± 0.160	0.376	0.296 ± 0.045	0.191 ± 0.054	0.281
CpG25	0.148 ± 0.020	0.295 ± 0.086	0.066	0.222 ± 0.043	0.202 ± 0.042	0.931
CpG26	0.202 ± 0.013	0.194 ± 0.107	0.571	0.188 ± 0.037	0.116 ± 0.023	0.356

Note: HPV16 +, HPV16-Positive; HPV16 -, HPV16-Negative; *P*-values were calculated by Mann-Whitney U-test; *P < 0.05.

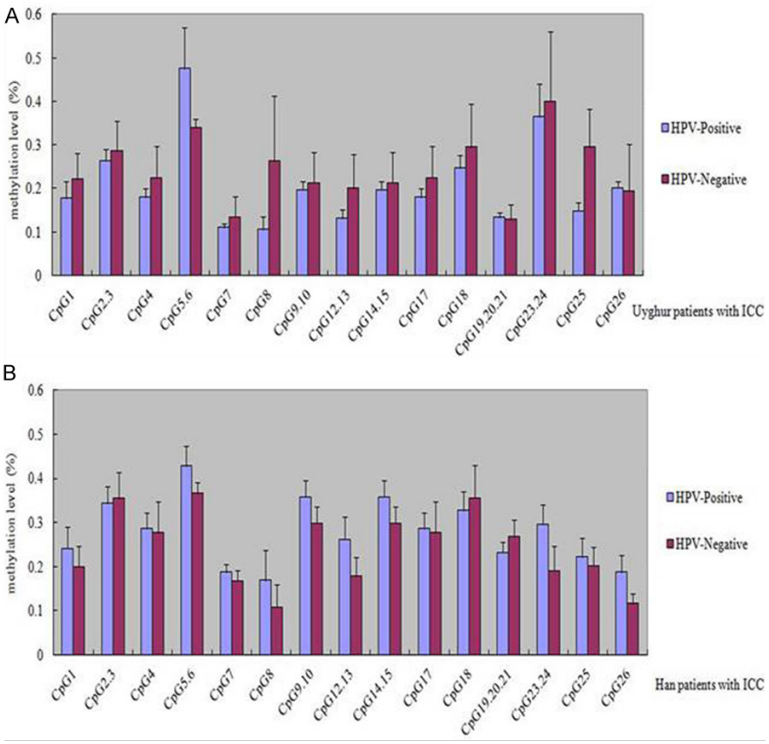

Figure 4. Methylation levels of *SFRP1* in cervical tissue samples from Uyghur and Han patients with and without HPV. The mean methylation of the CpG units of A. Uyghur and B. Han patients is presented for HPV-positive and HPV-negative individuals. *$P < 0.05$. Error bars represent standard deviation.

SFRP1 is suspected to play a crucial role in the cell cycle by modulating cell proliferation and cell growth [40] and may thus be considered a TSG. Most importantly, inactivation of *SFRP1* expression caused by aberrant promoter methylation may contribute to the activation of the Wnt signaling pathway that is highly significant for the control of cell proliferation [41, 42]. In general, *SFRP1* is considered an antagonist gene within the Wnt signaling pathway and has an essential effect on the suppression of tumor growth in several cancer entities [17, 43]. Overall, *SFRP1* is apparently able to downregulate the Wnt signaling pathway, and the frequent inactivation of the *SFRP1* gene in cancer highlights the importance of Wnt signaling in the pathogenesis of cervical cancer [17].

The aim of the present study was to assess the methylation status of CpG units of the *SFRP1* gene promoter in tissue samples from Uyghur and Han Chinese patients with different

cervical lesions. Our results show that the levels of methylation of CpG units in cervical cancer groups were significantly higher than in CIN2/3, CIN1, and normal cervical tissues groups. We also found a trend for increased methylation with the more serious lesions. These results are consistent with previous observations [18, 39]. The data confirm a significant association between the development of cervical cancer and *SFRP1* gene methylation and suggest that *SFRP1* could be used as a novel epigenetic biomarker to diagnose cervical carcinoma, especially as a noninvasive screening method.

The difference in methylation between ICC, CIN2/3, CIN1, and normal groups was of marginal statistical significance in Uyghur samples. Apart from CpG2.3, CpG4, and CpG17 units, the extent of methylation in the cervical cancer group was higher than that in the CIN2/3 group. There was no significant difference between any other two groups. Analysis of the above results leads to the following conclusions. First, methylation of the CpG units which chosen to amplified in the *SFRP1* gene, may not be the main reason for the formation of cervical cancer in Uyghur patients. *SFRP1* may have been functional since the evolution of cervical cancer, and the Wnt signaling pathway, may help maintain a normal state. Second, the Wnt signaling pathway may be involved in cervical oncogenesis. However, methylation was not the mechanism of the decreased expression of the *SFRP1* gene. Additional studies are needed to determine whether methylation of the *SFRP1* gene is involved in the development of cervical carcinogenesis in Uyghur patients.

Analysis of cervical cancer samples from Han patients showed that methylation was higher within CpG1, CpG7, CpG9.10, CpG12.13, CpG14.15, CpG18, and CpG25 units of the *SFRP1* promoter than in CIN2/3 ($P < 0.05$). Together, these findings indicate that methylation of the *SFRP1* gene promoter might be involved in the progression from CIN2/3 to cervical cancer through the activation of Wnt signaling pathway. Furthermore, methylation of these units might be used to detect cervical carcinoma.

The MassARRAY data indicated that methylation of *SFRP1* was much higher in Han populations than in the Uyghur populations with cervical cancer. The methylation of the same CpG units in comparable tissue was generally lower in the Uyghur populations than in the Han populations. In addition, we found significant differences in the frequency of *SFRP1* methylation at CpG1, CpG2.3, CpG4, CpG8, CpG17, CpG19.20.21, and CpG26 in Uyghur populations between the four cervical groups. Conversely, the methylation of *SFRP1* in Han populations at CpG1, CpG7, CpG9.10, CpG12.13, CpG14.15, CpG18, and CpG25 exhibited significant differences between the four groups. These observations of different methylation of *SFRP1* among Han and Uyghur populations suggest there are ethnic differences in genetic susceptibility to cervical cancer.

Persistent infection with HR-HPV is necessary, but not sufficient, for causing cervical cancer. Cervical carcinogenesis is a complex process whose etiology remains unclear and whose clinical prognosis remains poor. RASSF1A, which acts as a tumor suppressor gene, has no distinct correlation with HPV infection in cervical cancer [44]. Kuzmin et al. [45] reported that methylation of a tumor suppressor gene, and HR-HPV infection, are two independent events. In our study, no significant differences were found between the methylation level of the *SFRP1* gene and HPV16 infection in Uyghur and Han patients with cervical squamous cell carcinoma ($P > 0.05$). This suggests that HR-HPV16 infection is unrelated to *SFRP1* gene methylation, similar to previous findings [44, 45].

The current study is somewhat limited because the sample size is too small to yield more accurate results. In addition, other experiments, such as real-time PCR, cannot be performed after MassARRAY because of insufficient remaining tissue. Finally, because of limited economic and cultural development in minority regions of China, follow-up visits to the Uyghur population were problematic. Nevertheless, the results of this study provide support for further investigation on the etiology of cervical cancer in different ethnic groups.

In conclusion, this is the first study to demonstrate that *SFRP1* CpG island hypermethylation-mediated silencing of *SFRP1* is related to cervical cancer in Han and Uyghur populations, and to show significant differences between the two ethnic groups. We also found that there were no correlations between the methylation of *SFRP1* and infection with HPV16 in Uyghur

and Han patients with cervical carcinoma. This suggests that several specific methylation sites of *SFRP1* might serve as a biomarker for the early diagnosis of cervical cancer and may also reflect ethnic differences in genetic susceptibility to this disease. Most importantly, reducing the methylation of *SFRP1* may provide a mechanism-based target for new therapies for patients with cervical carcinoma.

Acknowledgements

This work was supported by the Preeminent Youth Foundation of Shihezi University School of Medicine (No.2013ZRKXYQ-YD18). The funding agencies had no role in the design, execution, or analysis of the data from this study, or the decision to submit this paper for publication.

Disclosure of conflict of interest

None.

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Supplementary Table 1. The prevalence of cervical cancer in Han and Uyghur samples (%)

Ethnicity	Cancer	Control	Total	χ^2	*P*
Han	58 (32.4)	121 (67.6)	179	4.368	0.037*
Uyghur	54 (44.3)	68 (55.7)	122		

Note: Control: CIN2/3, CIN1 and normal; χ^2, *P*: cancer with control; I; *: $P < 0.05$.

Supplementary Table 2. Sequences of PCR primers used in this study

Gene	Primer	Sequence (5,-3,)
SFRP1	For	5'-GTTTTATTTTGGGGTTTGGAGGTTT-3'
	Rev	5'-ACAAAAAAATAATACTACCCAACCTA-3'
HPV16	For	5'-GACCCAGAAAGTTACCACAG-3'
	Rev	5'-CACAACGGTTTGTTGTATTG-3'
β-globin	For	5'-CAACTTCATCCACGTTCACC-3'
	Rev	5'-GAAGAGCCAAGGACAGGTAC-3'

Note: "For": Forward, "Rev": Reverse.