Secreted frizzled-related protein promotors are hypermethylated in cutaneous squamous carcinoma compared with normal epidermis

Junqin Liang¹, Xiaojing Kang¹, Yilinuer Halifu¹, Xuewen Zeng², Tianbo Jin³, Mingxia Zhang³, Dong Luo¹, Yuan Ding¹, Yunmin Zhou¹, Buwajier Yakeya¹, Dilinuer Abudu¹ and Xiongming Pu¹*

Abstract

Background: The Wnt signaling pathway is abnormally activated in many human cancers. Secreted frizzled-related proteins (SFRPs) function as negative regulators of Wnt signaling and play an important role in carcinogenesis. SFRP promoter hypermethylation has often been identified in human cancers; however, the precise role of SFRPs in cutaneous squamous cell carcinoma (SCC) is unclear.

Methods: The methylation status of the SFRP family was analyzed in an age-and sex-matched case-control study, including 40 cutaneous SCC cases and 40 normal controls, using the MassARRAY EpiTYPER system.

Results: The methylation rate of SFRP1, SFRP2, SFRP4, and SFRP5 promoters was significantly higher in cutaneous SCC tissues than in adjacent tissue and normal skin samples.

Discussion: Our manuscript mainly discussed the average methylation rate of SFRPs (SFRP1, SFRP2, SFRP4, and SFRP5) promoters are significantly high in tumor tissue samples and the average CpG island methylation rate among different pathological levels of cutaneous SCC between these genes are different.

Conclusions: Our findings suggest that promoter hypermethylation of SFRPs is associated with the development of carcinoma, and could be a useful tumor marker for cutaneous SCC and other types of cancers.

Keywords: Cutaneous squamous cell carcinoma, Signaling pathway, DNA methylation, SFRP

Background

Non-melanoma skin carcinomas, comprising cutaneous squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), are the most common malignancies in fair-skinned Caucasians [1]. Unlike almost all basal cell carcinomas, cutaneous SCCs are associated with a substantial risk of metastasis [2]. The etiology of cutaneous SCC is multifactorial, with both environmental and host factors being important. However, exposure to ultraviolet radiation is the most common cause [3, 4]. Other risk factors include impaired immune surveillance, as seen in organ transplant recipients, and human papillomavirus infections [5].

The Wnt family of proteins includes a wide range of secreted growth factors that regulate cell differentiation, proliferation, migration, and organogenesis during embryonic development [6]. Aberrant activation of the Wnt pathway has been observed to inhibit tumor cell apoptosis during human carcinogenesis [7, 8]. Wnt signaling is activated on binding of the Wnt ligand to the frizzled membrane receptor [9], and a family of five secreted frizzled-related glycoproteins (SFRP1-5) has been identified as modulators of Wnt signaling [10]. Moreover, the epigenetic silencing of SFRP genes, leading to oncogenic activation of the Wnt pathway and tumor progression was reported in many human cancers [11–14].

Although the involvement of Wnt signaling in carcinogenesis has been extensively studied, the associations of the SFRP family with tumorigenesis have only recently begun to be explored. Previous studies have reported SFRP downregulation in colorectal cancer, gastric cancer [15], and invasive breast tumors [16, 17]. Additionally, Wnt5a signaling was found to contribute to tissue invasion by non-melanoma skin cancer, including both SCC.
family members, including test. All P < 0.05 = 40) and adjacent tissues

μ SFRP1 (2015) 15:641 N SFRP4 SFRP1 SFRP5 = 40) were collected from the face or the hands/legs H = 40) Control (H = 40) were obtained from the head or the hands/legs of the 40 cutaneous SCC patients. Normal skin samples (n = 40) were collected from the face or the hands/legs of the 40 controls. This study was approved by the ethics committee of the People's Hospital of Xinjiang Uygur Autonomous Region. Written informed consent was also obtained from each participant enrolled in the study.

Methods
Study populations and tissue samples
To investigate the methylation status of the SFRP family, we conducted an age- and gender-matched case-control study including 40 cutaneous SCC cases and 40 normal controls. The patients were recruited from the Dermatology Department of the People's Hospital of Xinjiang Uygur Autonomous Region (Xinjiang, China), and had received a diagnosis of histologically confirmed cutaneous SCC between January 2012 and February 2014. Controls were normal individuals from the Plastic Surgery Department of the hospital who reported no history of any type of cancer at the time of study recruitment. The basic characteristics for the participants included in this study are presented in Table 1. Cutaneous SCC tissues (n = 40) and adjacent tissues (n = 40) were obtained from the head or the hands/legs of the 40 cutaneous SCC patients. Normal skin samples (n = 40) were collected from the face or the hands/legs of the 40 controls. This study was approved by the ethics committee of the People's Hospital of Xinjiang Uygur Autonomous Region. Written informed consent was also obtained from each participant enrolled in the study.

DNA extraction, sodium bisulfite modification, and PCR
Genomic DNA from tissue samples was extracted using the Tissue DNA Kit (Qiagen) according to the manufacturer’s recommendations. The DNA concentration and quality were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Houston, TX). Bisulfite modification of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer’s protocol. Bisulfite-treated genomic DNA was amplified in a 384-well plate using HotStar Taq Polymerase in a 5-μl reaction volume (Qiagen). PCR conditions were 94 °C for 4 min followed by 45 cycles of 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 60 s, with a final extension of 72 °C for 3 min. Primer sequences are given in Table 2.

Quantitative DNA methylation analysis by MassARRAY EpiTyper
The PCR products were treated according to the standard protocol (Sequenom EpiTyper Assay) by SAP treatment and T-cleavage reaction. The samples were then cleaned by Resin and were dispensed to a 384 SpectroCHIP by Nanodispenser. DNA methylation levels were determined as previously described [20] using MassARRAY EpiTYPER (SEQUENOM Inc., Herston, QLD, Australia) system, which is based on MALDI-TOF MS [21]. The mass spectrum was collected by MassARRAY Spectrometer and analyzed by EpiTYPER v.1.0 software (SEQUENOM).

Statistical analysis
The SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) were used for statistical analysis. The methylation levels in cutaneous SCC patients and normal samples were compared by nonparametric Mann-Whitney U test or the Kruskal-Wallis H test. All P-values presented in this study were two sided, and we used P < 0.05 as the cutoff value for statistical significance. The receiver operating characteristic (ROC) curve was used to estimate the specificity, sensitivity, and accuracy of the regression test.

Results
In the present study, we investigated the methylation status of SFRP1, SFRP2, SFRP4, and SFRP5 in 40 cutaneous SCC patients and 40 normal subjects. Table 1 showed the distribution of age, sex, and pathological level among cases and controls. The mean age for the case group was 67.11 ± 9.24 years and 64.31 ± 8.91 years for the control group. There were no significant differences in age and sex distribution between the case and control groups (p > 0.05).

We investigated the promoter methylation rate of SFRP1, SFRP2, SFRP4, and SFRP5 in 40 cutaneous SCC tissues, 40 adjacent tissues, and 40 normal samples using the MassARRAY EpiTyPER system. Aberrant promoter methylation of SFRP1, SFRP2, SFRP4, and SFRP5 was detected in cutaneous SCC tissues and adjacent tissues compared with normal samples. Additionally, the frequency of
### Table 2 Primer sequences for amplifying SFRP genes

| Gene | Forward | Reverse | bp | CpGs | Tm |
|------|---------|---------|----|------|----|
| SFRP1 | SFRP1_1 | aggaagagagTATGTGTGTATGAGATGATTGGTTTT | cagtaatactactataaggaggactAAACACCTCTCTAAAAATGGG | 290 | 7 | 56 |
| SFRP1 | SFRP1_2 | aggaagagagAGTTGGGAGGTTGAAGGGAGATTGA | cagtaatactactataaggaggactAAACACCTCTCTCTAAACCC | 300 | 12 | 56 |
| SFRP2 | SFRP2_1 | aggaagagagGGTTAGGTGTGTGTTTTTTTTTT | cagtaatactactataaggaggactCACAACCAAAATTTCTTTTTTT | 235 | 9 | 56 |
| SFRP2 | SFRP2_2 | aggaagagagGGGGATGATGAGATTATTATT | cagtaatactactataaggaggactCACAACCAAAATTTCTTTTT | 296 | 9 | 56 |
| SFRP4 | SFRP4_1 | aggaagagagGTATGTGTGTGTGTTGTTTTTT | cagtaatactactataaggaggactTCCCAACTACAACCAATTTTA | 314 | 6 | 56 |
| SFRP4 | SFRP4_2 | aggaagagagGTATGTGTGTGTGTTGTTTTTT | cagtaatactactataaggaggactTCCCAACTACAACCAATTTTA | 390 | 36 | 56 |
| SFRP5 | SFRP5_1 | aggaagagagGTATGTGTGTGTGTTGTTTTTT | cagtaatactactataaggaggactTCCCAACTACAACCAATTTTA | 323 | 6 | 56 |
| SFRP5 | SFRP5_2 | aggaagagagGTATGTGTGTGTGTTGTTTTTT | cagtaatactactataaggaggactTCCCAACTACAACCAATTTTA | 329 | 6 | 56 |

SFRP_1 and SFRP_2 refer to two amplified fragments of SFRP
**Discussion**

Cutaneous SCC is the second most common cancer among Caucasians [2]. Although it has been suggested to arise through the accumulation of multiple genetic changes involving a complex multi-step process [1], the precise pathogenesis remains unclear. However, numerous studies have recently shown that the epigenetic dysregulation of tumor suppressor genes could serve as a biomarker to predict the diagnosis and prognosis of human disease and malignancy [23–25].

In the present study, we describe the aberrant DNA methylation of four members of the **SFRP** family, **SFRP1**, **SFRP2**, **SFRP4**, and **SFRP5**, in cutaneous SCC in a Chinese population. Methylation of these genes has been reported previously in lung cancer, prostate cancer, colorectal cancer [26], and human glioblastoma [27]. Additionally, several studies have reported the lack of methylation in nonmalignant tissues and described methylation as a tumor-restricted event [23, 28, 29]. Here, we showed that frequent promoter hypermethylation of **SFRPs** was significantly elevated in cutaneous SCC samples compared with adjacent tissues and control samples. Interestingly, we also observed marked differences in the levels of aberrant DNA methylation between these genes (**SFRP1**, **SFRP2**, **SFRP4**, and **SFRP5**), suggesting that hypermethylation of these **SFRPs**, particularly of **SFRP1**, could be important in the onset of cutaneous SCC.

Aberrant hypermethylation of CpG islands in gene promoters was previously shown to be a primary mechanism in the inactivation of several tumor suppressor genes [30]. We assessed the methylation status of the CpG islands in **SFRP** promoters in different pathological levels of cutaneous SCC tissue, and found the methylation rate to increase with the pathological level. ROC curve analysis showed that the **SFRP1** CpG site is a potential biomarker of cutaneous SCC, and the further identification of genes that are methylated during cutaneous SCC carcinogenesis together with an improvement in the quantitative rather than the qualitative analysis of methylation will be of great value in the future molecular screening of cutaneous SCC.

The potential importance of **SFRP** silencing in cutaneous SCC relates to its involvement in the Wnt signaling pathway. This pathway is involved in the tumorigenesis of many human cancers, including those of the colon, breast, lung, and liver, as well as melanomas [7]. Chung et al. also recently demonstrated the frequent aberrant methylation of **SFRPs** in cervical SCC [31]. Additionally, two further studies indicated that frequent aberrant methylation of **SFRPs** occurs in hepatocellular carcinoma, and that restoration of **SFRP1** attenuates Wnt signaling, decreases the abnormal accumulation of β-catenin in the nucleus, and suppresses cell growth [32, 33]. To date, however, the regulation of the Wnt pathway in cutaneous SCC has remained unclear. Pourreyron et al. [18] demonstrated that Wnt5a is overexpressed in non-melanoma skin cancer, which contributes to tissue invasion. Additionally, the study presented here also suggests the upregulation of **SFRP1** and **SFRP2** expression in cutaneous SCC, in particular **SFRP2**, exhibit very high constitutive expression in normal skin. The data provided by the Human Protein Atlas on cutaneous SCC (www.proteinatlas.org) showing that the **SFRPs** (**SFRP1**, **SFRP2**, **SFRP4**, and **SFRP5**) exhibit very low expression in cancer tissues, except **SFRP1**, which was slightly high. Our results showed that the **SFRPs** were highly methylated in cutaneous SCC, which in accordance with the data of the

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**Table 3** Methylation frequency (%) comparison of **SFRP** CpG sites in three tissue groups

| Genes | No. of CpG islands | Frequency of promoter methylation (%) |
|-------|--------------------|--------------------------------------|
|       | T vs. N | T vs. A | A vs. N |
| SFRP1 | 17      | 82.35 (14/17) | 58.82 (10/17) | 29.41 (5/17) |
| SFRP2 | 14      | 50 (7/14) | 57.14 (8/14) | 42.86 (6/14) |
| SFRP4 | 18      | 72.22 (13/18) | 72.22 (13/18) | 61.11 (11/18) |
| SFRP5 | 11      | 54.54 (6/11) | 54.54 (6/11) | 54.54 (6/11) |

*P* value <0.05

| Test | Tumor tissue | Adjacent tissue | Normal samples |
|------|--------------|----------------|---------------|
| Mann-Whitney U | P* value <0.05 | <0.05 | <0.05 |
Human Protein Atlas on cutaneous SCC. Nevertheless, much work remains to be done to obtain a clearer understanding of the role of SFRPs in Wnt signaling and tumor pathogenesis.

Conclusions
In conclusion, our study demonstrates that promoter hypermethylation of SFRPs (SFRP1, SFRP2, SFRP4, and SFRP5) is associated with the development of cutaneous SCC. Although it is not possible to determine whether SFRPs are directly related to carcinogenesis from this study, the aberrant methylation of SFRPs may nevertheless be a useful tumor biomarker for early diagnosis. Despite the current study possessing enough statistical power, some limitations should be considered. First, we limited our investigation to only four SFRPs: SFRP1, SFRP2, SFRP4, and SFRP5. Therefore, we cannot exclude the possibility that other genes may play a role in the pathogenesis of cutaneous SCC. Second, the sample size of our study was relatively small, so our findings should be confirmed in larger case-control studies.

Table 4 Methylation frequency (%) of SFRP CpG islands at different cutaneous SCC pathological levels

| Gene  | CpG Site | Stage I  | Stage II | Stage III | H   | P-value |
|-------|----------|----------|----------|-----------|------|---------|
| SFRP1 | CpG1_5   | 0        | 0        | 9         | 6.157| 0.024*  |
|       | CpG1_7   | 9        | 6        | 2         | 5.103| 0.031   |
|       | CpG2_1   | 7        | 0        | 2         | 2.136| 0.021   |
|       | CpG2_8   | 5        | 7        | 4         | 8.514| 0.017   |
| SFRP2 | CpG1_5   | 44       | 29       | 19        | 6.142| 0.015   |
|       | CpG2_1   | 47       | 48       | 17        | 6.136| 0.023   |
|       | CpG2_3.4 | 55       | 42       | 18        | 5.218| 0.015   |
| SFRP4 | CpG1_3   | 2        | 3        | 2         | 7.158| 0.009   |
|       | CpG2_2   | 9        | 2        | 5         | 7.156| 0.023   |
| SFRP5 | CpG1_5   | 40       | 33       | 18        | 5.54 | 0.031   |
|       | CpG2_1   | 32       | 19       | 34        | 3.136| 0.021   |

N: number of samples; H: the Kruskal-Wallis H test
*p ≤ 0.05 indicates statistical significance

Fig. 1 Comparison of average CpG island methylation rate in the SFRP gene family. T, tumor tissues; A, adjacent to carcinoma tissue; N, normal tissue samples
Fig. 2 Receiver operating characteristic (ROC) curves for six CpG sites in the SFRP1 promoter. The ROC curves plot sensitivity and 1-specificity. Areas under the curve (AUC) and $P$ values were shown in the graph.
Abbreviations
SCC: Squamous cell carcinoma; BCC: Basal cell carcinoma; SFRPs: Secreted frizzled-related proteins; ROC: Receiver operating characteristic; AUC: Area under the curve.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JL, XK, and YH designed the study, carried out the molecular genetic studies, participated in the statistical analysis and drafted the manuscript. XZ, TJ, and MZ participated in the molecular genetic studies and statistical analysis. DL, YD, and YZ participated in the design of the study and performed the statistical analysis. BY and DA conceived the study, participated in its design and coordination, and helped to draft the manuscript. XP conceived the study, participated in its design and coordination, and funded the study. All authors read and approved the final manuscript.

Authors’ information
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Author details
1Department of Dermatology, The People’s Hospital of Xinjiang Uyghur Autonomous Region, Urumqi 830000, China. 2Department of Plastic surgery, The People’s Hospital of Xinjiang Uyghur Autonomous Region, Urumchi 830000, China. 3School of Life Sciences, Northwest University, Xi’an 710069, China. National Engineering Research Center for Miniaturized Detection Systems, Xi’an 710069, China.

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