Original Article

Hypermethylation of TFPI2 correlates with cervical cancer incidence in the Uygur and Han populations of Xinjiang, China

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Abstract: Tissue factor pathway inhibitor 2 (TFPI2) is a Kunitz-type serine protease inhibitor, which plays an important role in the etiology of human malignancies. DNA methylation is a common epigenetic modification of the genome that is involved in regulating many cellular processes. In addition to human papilloma virus (HPV) infection, DNA methylation may play a role in the carcinogenesis of cervical cancer. Methylation of 22 CpG sites in the promoter region of the TFPI2 gene was detected by MassARRAY spectrometry and a gene mass spectrogram was drawn using MALDI-TOF MS. HPV16 was detected by PCR. We show that aberrant methylation of TFPI2 is present in a higher proportion of invasive cervical carcinoma (ICC) clinical samples as compared to normal cervical samples in Uygur and Han. Across the four pathologic lesions of the progression of cervical cancer, ICC showed the highest level of aberrant methylation, and with a stronger correlation between CpG site and lesion grade in Uygur than in Han. Moreover, a difference in TFPI2 methylation between Uygur patients positive and negative for HPV16 infection was observed at CpG_6 (P = 0.028) and CpG_15 (P = 0.007). Altogether, these results indicate that DNA methylation of TFPI2 may play an important role in the carcinogenesis of cervical cancer and that the differential methylation of TFPI2 may at least partially explain the disparity in cervical cancer incidence between Uygur and Han women.

Keywords: TFPI2, HPV16, cervical cancer, Uygur, Han, methylation

Introduction

Cervical cancer remains a major cause of cancer mortality in women worldwide. According to GLOBOCAN estimates, approximately 528,000 patients were diagnosed with cervical cancer in 2012 (GLOBOCAN 2012). In China, there are approximately 130,000 new cases diagnosed every year, accounting for one third of the total new cases worldwide, with 53,000 deaths attributed to the cervical cancer annually, one fourth of the total deaths worldwide [1]. Kashi, Xinjiang, inhabited by a large number of Uygur, is an area of high cervical cancer incidence in China. According to a recent report, the incidence of cervical cancer in Uygur was 622/100,000 in Xinjiang in 2008 [2], much higher than the 126.94/100,000 incidence in the Han [3].

Testing for high-risk human papilloma virus (HPV) may help to triage patients with pre-invasive disease and determine appropriate clinical intervention. However, HPV presence/absence does not solely dictate the molecular status of the cervical epithelial cell. Persistent infection with high-risk types of human papillomavirus (HR-HPV) is known to cause cervical cancer; however, additional genetic alterations are also required for disease progression [4]. DNA methylation is an important epigenetic modification of the genome that is involved in regulating many cellular processes, including embryonic development, transcription, chromatin struc-
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Materials and methods

Patients

Multistage cluster sampling was utilized to randomly select 302 cases of Han and Uygur patients diagnosed with histologically confirmed ICC (55 cases of Uygur, 58 cases of Han), CIN (CIN2/3: 36 cases of Uygur, 57 cases of Han; CIN1: 11 cases of Uygur, 36 cases of Han) and normal cervical tissues (21 cases of Uygur, 28 cases of Han). All patients were referred from 2001 to 2009 and obtained from the 12th Army Hospital in Kashi, Xinjiang, Kashi District People’s Hospital, and Shihezi University School of Medicine. The samples from the normal group in these two nations were diagnosed with no cervical lesions, obtaining after total hysterectomy in patients with uterine fibroids. Surgical excision was performed according to routine protocols of clinical care. Hematoxylin-eosin and immunohistochemistry staining of slides prepared from paraffin-embedded tissue was used to confirm the diagnosis of each case. Each sample was confirmed by two experienced pathologists according to the WHO Pathology & Genetics Tumours of the Breast and Female Genital Organs (seventh edition).

DNA extraction, purification, and bisulfate modification

An EZ DNA methylation process kit was purchased from Zymo Research, Irvine, CA; PCR MassCLEAVE Reagent kit was purchased from Sequenom, San Diego, CA. 5×TBE dNTPs (10 mM), and the PUC19DNA/Msp (Hpa II) marker were obtained from Sangon Biotech Company, Shanghai. Genomic DNA was previously extracted from paraffin-embedded tissue using a phenol-chloroform protocol and resuspended. Quantification of total DNA was performed using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). DNA was bisulfite treated using the EZ DNA Methylation Kit (Zymo Research) and eluted in buffer [11]. One microliter of bisulfite-treated DNA was used as a template for methylation quantification with PCR. Primers for each marker were designed to target the bisulfite-modified methylated sequences of each gene. The primer sequence for the TFPI2 gene was designed using EpiDesigner (http://www.epidesigner.com; Sequenom) while primers for β-globin and HPV16 were obtained from literature [12]. The primer se-
TFPI2 methylation in cervical cancer

Figure 1. DNA methylation status of TFPI2 in Uygur patients. A. Clustering analysis diagram of ICC, CIN2/3, CIN1 and normal lesion grades in Uygur. Yellow shows that the methylation rate is 100%, red shows 0%, gray indicates areas that have not been analyzed. Each line represents one CpG site, and each column represents one lesion grade. Clustering analysis diagram showing positive correlation of methylation level with lesion grade. B. A histogram depicting the methylation level of every CpG site with differences between the four lesion grades and the trend of the methylation indicated. C. Clustering analysis diagram in Han. Clustering analysis diagram showing positive correlation of methylation level with lesion grade. D. The histogram indicates the difference between the four lesion grades in Han. *P < 0.05, **P < 0.01, ***P < 0.001.
sequences for the β-globin cDNA were as follows: β-globin-RT-forward: 5'-CAACTTCATCCACGTTC-ACC-3', and β-globin-RT-reverse: 5'-GAAGAGCAAGGACAGGTAC-3', generating a 268-bp PCR product. The primer sequences for the TFPI2 cDNA were: TFPI2-RT-forward: 5'-aggaagagA-GGGGTTAGGGAGATTAGATAAGTT-3', and TFPI2-RT-reverse: 5'-cagtaatacgactcactatagggagaaggctCAAAAATAACTACACCCACACCTTC-3', generating a 354-bp PCR product. The primer sequences for the HPV16 cDNA were: HPV16-RT-forward: 5'-GACCCAGAAAGTTACCACAG-3', and HPV16-RT-reverse: 5'-CACAACGGTTTGT-TGTATTG-3', generating a 268-bp PCR product. PCR conditions were 94°C for 4 minutes, 35 cycles of 94°C for 45 seconds/58°C for 45 seconds/72°C for 1 minute, and 72°C for 10 minutes. PCR products were resolved through 2% agarose gels, visualized using a transilluminator, and then analyzed by pyrosequencing using the Biotage Sample Prep kit and the forward primer for sequencing.

**Quantitative MassARRAY analysis of gene methylation status**

A nanodispenser was used to distribute 22 nL of the pyrolysis reaction liquid to a SpectroCHIP (Sequenom), which was loaded with the reaction substrate. Gene mass spectrometry were acquired by MassARRAY (Bruker-Sequenom) using MALDI-TOF MS and analyzed using Epityer® software (v1.05). Methylation detection provides the intensity of methylation of every CpG site in the TFPI2 gene, which appears as different colors. A red-to-yellow gradient equals sample methylation intensity from 0% to 100%, as in Figure 1A. CpG island sequences were obtained from the UCSC website (http://www.genome.ucsc.edu).

**Statistical analysis**

The TFPI2 methylation data were analyzed using Epityerv1.05 software, which reports a P value for each CpG site based on a comparison of the mean methylation level in each of the four groups. A Kruskal-Wallis H test was used to compare the DNA methylation status of TFPI2 between the four groups in Uygur or Han patients. A Student-Newman-Keuls analysis was used to analyze the difference between two groups in Uygur or Han patients. A student’s t-test was used to compare the TFPI2 methylation level between the Uygur and Han patients. We assessed associations between TFPI2 methylation at each CpG site and lesion grade using the Spearman rank correlation. Descriptive data about HPV16 infection were analyzed using Chi-square (X^2) and t-tests were used for comparisons of DNA methylation levels between patients positive

<table>
<thead>
<tr>
<th>Sites</th>
<th>Lesion Grades</th>
<th>Normal</th>
<th>CIN1</th>
<th>CIN2/3</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG_1</td>
<td>Normal</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>CIN1</td>
<td>0.873</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>CIN2/3</td>
<td>0.946</td>
<td>0.826</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.019*</td>
<td>0.047*</td>
<td>0.013*</td>
<td></td>
</tr>
<tr>
<td>CpG_15</td>
<td>Normal</td>
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<td></td>
<td>CIN1</td>
<td>0.696</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN2/3</td>
<td>0.57</td>
<td>0.963</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.003**</td>
<td>0.052</td>
<td>0.008**</td>
<td></td>
</tr>
<tr>
<td>CpG_18.19</td>
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<td></td>
<td></td>
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<td></td>
<td>CIN1</td>
<td>0.244</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN2/3</td>
<td>0.274</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.000***</td>
<td>0.068</td>
<td>0.004**</td>
<td></td>
</tr>
<tr>
<td>CpG_20</td>
<td>Normal</td>
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<td></td>
<td>CIN1</td>
<td>0.267</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CIN2/3</td>
<td>0.527</td>
<td>0.523</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.008**</td>
<td>0.379</td>
<td>0.034*</td>
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</tr>
<tr>
<td>CpG_23</td>
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<td>0.586</td>
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<td>CIN2/3</td>
<td>0.497</td>
<td>0.971</td>
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<tr>
<td></td>
<td>ICC</td>
<td>0.009**</td>
<td>0.178</td>
<td>0.046*</td>
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<tr>
<td>CpG_24,25,26</td>
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<td>1</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>CIN11</td>
<td>0.79</td>
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<tr>
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<td>CIN2/3</td>
<td>0.935</td>
<td>0.732</td>
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</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.024*</td>
<td>0.041*</td>
<td>0.019*</td>
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</tr>
<tr>
<td>CpG_31</td>
<td>Normal</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN12</td>
<td>0.488</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>CIN2/14</td>
<td>0.665</td>
<td>0.706</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.000***</td>
<td>0.023*</td>
<td>0.000***</td>
<td></td>
</tr>
</tbody>
</table>

Note: There is a significant difference between ICC and other lesion grades in these CpG sites in the Uygur cohort. Student-Newman-Keuls analysis of variance between different lesion grades in Uygur. *P < 0.05, **P < 0.01, ***P < 0.001.
**Table 2. Pairwise comparison of lesion grades in the Han cohort**

<table>
<thead>
<tr>
<th>Sites</th>
<th>Lesion Grades</th>
<th>Normal</th>
<th>CIN1</th>
<th>CIN2/3</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG_1</td>
<td>Normal</td>
<td>1</td>
<td>0.913</td>
<td>1</td>
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<tr>
<td></td>
<td>CIN1</td>
<td>0.973</td>
<td>0.877</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN2/3</td>
<td>0.005**</td>
<td>0.003**</td>
<td>0.002**</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.364</td>
<td>0.009**</td>
<td>0.045*</td>
<td>1</td>
</tr>
<tr>
<td>CpG_2.3.4.5</td>
<td>Normal</td>
<td>1</td>
<td>0.115</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN1</td>
<td>0.369</td>
<td>0.397</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN2/3</td>
<td>0.346</td>
<td>0.23</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.013*</td>
<td>0.007**</td>
<td>0.066</td>
<td>1</td>
</tr>
<tr>
<td>CpG_20</td>
<td>Normal</td>
<td>1</td>
<td>0.733</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN1</td>
<td>0.759</td>
<td>0.955</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN2/3</td>
<td>0.003**</td>
<td>0.004**</td>
<td>0.003**</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.012*</td>
<td>0.018*</td>
<td>0.019*</td>
<td>1</td>
</tr>
<tr>
<td>CpG_21.22</td>
<td>Normal</td>
<td>1</td>
<td>0.815</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN1</td>
<td>0.852</td>
<td>0.23</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN2/3</td>
<td>0.659</td>
<td>0.807</td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td>ICC</td>
<td>0.012*</td>
<td>0.018*</td>
<td>0.019*</td>
<td>1</td>
</tr>
<tr>
<td>CpG_23</td>
<td>Normal</td>
<td>1</td>
<td>0.683</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIN1</td>
<td>0.415</td>
<td>0.704</td>
<td>1</td>
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<tr>
<td></td>
<td>CIN2/3</td>
<td>0.003**</td>
<td>0.01*</td>
<td>0.015*</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: There is a significant difference between ICC and other lesion grades for most CpG sites in the Han cohort. Student-Newman-Keuls analysis of variance between different lesion grades in the Han Chinese. *P < 0.05, **P < 0.01.

and negative for HPV16 infection. Observed differences with a P value of < 0.05 were considered statistically significant.

**Results**

**DNA methylation status of TFPI2 in Uygur patients**

Based on the clustering analysis of ICC (n = 36), CIN2/3 (n = 21), CIN1 (n = 8), and normal pathology (n = 16) samples from Uygur patients, we generated a heat map, which demonstrates an increased rate of methylation in the ICC samples as compared to the other lesion classifications (Figure 1A). To determine if there was a trend in the methylation present in these lesion grades, we compared methylation at each CpG site via a histogram. As shown in Figure 1B, the methylation level is significantly different between ICC samples and the other lesion classifications at CpG_1, CpG_15, CpG_18.19, CpG_20, CpG_23, CpG_24,25,26, and CpG_31. P values of each comparison are provided in the supplementary material, Table S1. Moreover, methylation of the TFPI2 gene in ICC is higher than in CIN2/3, CIN1, and the normal pathology groups (Figure 1B).

Next, we performed a pairwise comparison of the four lesion classifications in the Uygur cohort. Striking differences were observed between ICC and the other lesion classifications at CpG_1, CpG_2, CpG_21.22, CpG_23, and CpG_31. Differential TFPI2 methylation levels were also found between ICC and either the normal pathology samples or CIN2/3 at CpG_15, CpG_18.19, CpG_20 and CpG_23 (Table 1).

**DNA methylation status of TFPI2 in Han patients**

Based on the clustering analysis of ICC (n = 33), CIN2/3 (n = 38), CIN1 (n = 26), and normal pathology (n = 25) samples from Han patients, the TFPI2 methylation level was found to be correlated with cervical lesion grade (Figure 1C), with significant differences between the four lesion classifications at CpG_1, CpG_2, CpG_2.3.4.5, CpG_20, CpG_21.22, CpG_23, and CpG_31. (Figure 1D). P values are reported in the supplementary material, Table S2. Methylation of the TFPI2 gene was greater in the ICC group as compared to CIN1, CIN2/3, and normal pathology samples. Further, at CpG_21.22, CpG_23, and CpG_31, the methylation level increased with advancing pathology (Figure 1D).

Similar to the study in Uygur patients, we compared TFPI2 methylation between lesion grades in Han patients in a pairwise fashion. Interestingly, no difference was observed in the DNA methylation of TFPI2 between the normal pathology samples and CIN1 or CIN2/3, or between CIN1 and CIN2/3. At CpG_1, CpG_21.22, CpG_23, and CpG_31, a statistically
TFPI2 methylation in cervical cancer

Figure 2. Comparison of methylation in Han and Uygur across the four lesion grades. The X axis represents every CpG site; the Y axis represents methylation level. Presented are two examples of such a contrast as shown in Table 3 using a histogram. *P < 0.05.
**Table 3. Differences in the methylation levels of the TFPI2 gene**

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>t</td>
<td>1.026</td>
<td>0.88</td>
<td>0.471</td>
<td>1.322</td>
<td>1.85</td>
<td>1.177</td>
<td>1.511</td>
<td>1.034</td>
<td>0.271</td>
<td>0.541</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.311</td>
<td>0.387</td>
<td>0.641</td>
<td>0.195</td>
<td>0.072</td>
<td>0.247</td>
<td>0.14</td>
<td>0.308</td>
<td>0.788</td>
<td>0.592</td>
<td>0.124</td>
</tr>
<tr>
<td>CIN1</td>
<td>t</td>
<td>0.944</td>
<td>-1.94</td>
<td>0.473</td>
<td>-1.234</td>
<td>0.521</td>
<td>0.542</td>
<td>-0.861</td>
<td>-0.896</td>
<td>-0.646</td>
<td>-0.044</td>
<td>1.469</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.352</td>
<td>0.066</td>
<td>0.64</td>
<td>0.227</td>
<td>0.606</td>
<td>0.592</td>
<td>0.396</td>
<td>0.379</td>
<td>0.523</td>
<td>0.965</td>
<td>0.153</td>
</tr>
<tr>
<td>CIN2/3</td>
<td>t</td>
<td>1.24</td>
<td>0.856</td>
<td>1.319</td>
<td>0.142</td>
<td>0.346</td>
<td>1.657</td>
<td>-1.16</td>
<td>0.986</td>
<td>-0.485</td>
<td>0.258</td>
<td>2.078</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.22</td>
<td>0.398</td>
<td>0.193</td>
<td>0.161</td>
<td>0.731</td>
<td>0.105</td>
<td>0.251</td>
<td>0.329</td>
<td>0.63</td>
<td>0.798</td>
<td>0.043</td>
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<tr>
<td>ICC</td>
<td>T</td>
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<td>0.039</td>
<td>0.346</td>
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<tr>
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<td>P</td>
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<td>0.675</td>
<td>0.095</td>
<td>0.196</td>
<td>0.725</td>
<td>0.744</td>
<td>0.355</td>
<td>0.276</td>
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</table>

Note: Comparison of methylation levels of the TFPI2 gene between Han and Uygur cohorts in different lesion grades. There is a statistical difference only for CpG_31 of normal pathology samples and CpG_24.25.26 of CIN2/3 samples. A t-test was used to analyze the differences amongst CpG sites. P values were obtained from comparisons between Han and Uygur cohorts for the methylation levels of each CpG site in the four lesion grades. *P < 0.05.

**Table 4. Correlation of methylation of each CpG site and lesion grade in Uygur and Han cohorts**

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
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<td>Uygur</td>
<td>0.288***</td>
<td>0.304</td>
<td>0.108</td>
<td>0.266*</td>
<td>0.404***</td>
<td>0.247</td>
<td>0.413***</td>
<td>0.348**</td>
<td>0.301*</td>
<td>0.306**</td>
<td>0.231</td>
<td>0.456***</td>
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<td>Han</td>
<td>0.204*</td>
<td>0.118</td>
<td>0.059</td>
<td>0.164</td>
<td>0.058</td>
<td>0.239*</td>
<td>-0.007</td>
<td>0.281**</td>
<td>0.299**</td>
<td>0.191*</td>
<td>0.09</td>
<td>0.249**</td>
</tr>
</tbody>
</table>

Note: Uygur cohort has a stronger correlation between CpG site and lesion grade. Spearman rank correlation was used to analyze the relationship between CpG site and lesion grade. R value is correlation coefficient. *P < 0.05, **P < 0.01, ***P < 0.001.

**Table 6. Differential methylation levels between the four lesion grades at each CpG site relative HPV16 infection in cervical cancer**

<table>
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</thead>
<tbody>
<tr>
<td>t Uygur</td>
<td>-1.27</td>
<td>-1.81</td>
<td>-2.32*</td>
<td>-1.65</td>
<td>-2.89**</td>
<td>-0.05</td>
<td>-0.90</td>
<td>0.01</td>
<td>-1.15</td>
<td>-3.12**</td>
<td>-0.77</td>
<td>-1.50</td>
</tr>
<tr>
<td>Han</td>
<td>-1.04</td>
<td>-0.06</td>
<td>0.14</td>
<td>0.15</td>
<td>-0.95</td>
<td>-0.12</td>
<td>-0.41</td>
<td>0.08</td>
<td>-0.41</td>
<td>-0.41</td>
<td>0.07</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Note: Only three CpG sites are differentially methylated according to HPV16 infection in Uygur cervical cancer patients. A t-test was used to analyze difference among CpG sites. *P < 0.05, **P < 0.01.
significant difference ($P < 0.05$) was observed between ICC and the other lesion classifications. Increased methylation was observed in ICC relative to CIN1 and CIN2/3 at CpG_2.3.4.5 ($P = 0.009$ and $0.045$, respectively). Between ICC and normal pathology samples as well as ICC and CIN1, differences in methylation were also present at CpG20 ($P = 0.013$ and $0.007$, respectively) (Table 2).

Comparison of TFPI2 methylation between Han and Uygur patients amongst four lesion classifications

Although we observed similar TFPI2 methylation profiles between Uygur and Han patients, the Uygur have an increased incidence of cervical cancer as compared to the Han [13]. We hypothesized that Uygur women may have different genetic factors accounting for their increased susceptibility to ICC compared to Han women living in the same region. To address this question, we compared the differences between the TFPI2 DNA methylation profiles of the Uygur and Han patients. Of the CpG sites compared, only CpG_31 in the normal pathology samples and CpG_24.25.26 in the CIN2/3 samples were different between the two cohorts ($P = 0.044$ and $0.043$, respectively) (Table 3 and Figure 2C). In CIN1 and ICC groups, the differences in CpG methylation were not statistically significant (Figure 2C and 2D).

In order to confirm whether there is a correlation between TFPI2 methylation at every CpG site amongst the four lesion classifications, a Spearman analysis was performed. In the Uygur cohort, a correlation was found for CpG_1, CpG_12.13.14, CpG_15, CpG_18.19, CpG_20, CpG_21.22, CpG_23, and CpG_31, with strong statistical significance ($P < 0.001$) observed for CpG_1, CpG_15, CpG_18.19, and CpG_31 (Table 4). In contrast, in the Han cohort, such a correlation was only found for CpG_1, CpG_16.17, CpG_20, CpG_21.22, CpG_23, and CpG_31 (Table 4).

Association of cervical lesion grade with HPV16 infection

Based on a Chi-square test in the Uygur cohort, cervical lesion grade was associated with HPV16 infection, with an increased rate of positive HPV16 infections according to disease progression (Table S3). In contrast, the rate of positive HPV16 infections did not increase with disease progression in the Han cohort (Table S4), indicating that HPV16 infection may not be involved in the progression from CIN2/3 to cancer in Han patients. No further differences were observed between the Uygur and Han cohorts regarding HPV16 infection rate in cervical cancer (Table 5).

We also tested the difference in methylation levels between ICC patients positive and negative for HPV16 infection. In the Uygur samples, three CpG sites (CpG_6, CpG_15, and CpG_23) were found have increased methylation correlated with HPV16 infection (Table 6), while, in the Han samples, there was no significant association between any of the CpG sites and HPV16 infection (Table 6). Our observations indicate an association between TFPI2 gene methylation and HPV16 infection in Uygur cervical cancer patients.

Discussion

TFPI2 was previously identified as a tumor suppressor [14-16]; however, some studies have found TFPI2 hypermethylation in several cancer types [17], suggesting an important role for TFPI2 in the etiology of human malignancies. Accordingly, we also found a high level of TFPI2 methylation in cervical lesions with a trend of increased TFPI2 methylation from normal pathology to ICC. In the ICC group, almost every CpG site had a high level of methylation. We speculated that TFPI2 methylation is involved in cervical carcinogenesis.

We analyzed the methylation level of the TFPI2 gene in different lesion grades in Uygur and Han patient samples by a clustering analysis.
and observed a higher TFPI2 methylation level in ICC compared to the other lesion grades. Based on the results of the Kruskal-Wallis H test of ICC, CIN2/3, CIN1, and normal pathology samples, a statistically significant difference in TFPI2 methylation was found between the four lesion grades. A previous study reported that gene hypermethylation is associated with cervical cancer in Uygur women in Xinjiang [18]. Sova et al. found that the TFPI2 gene had a high frequency of aberrant methylation in ICC specimens [19]. The present study confirms these results by demonstrating TFPI2 methylation in cervical cancer. As we found a trend in increased TFPI2 methylation from normal pathology to ICC, we wanted to further elucidate the differences between lesion grades on the progression from normal pathology to ICC.

Next, we performed pairwise comparisons of TFPI2 methylation between the four lesion grades separately in the Uygur and Han cohorts, with a statistically significant difference ($P < 0.05$) observed between ICC and the other groups, especially at CpG_1, CpG_20, CpG_23, and CpG_31, which also were statistically significant between the two cohorts. These results demonstrate that DNA methylation of the TFPI2 gene may be involved in the tumorigenesis of cervical cancer.

We specifically focused on an area of high cervical cancer incidence in Xinjiang, China inhabited by a large number of Uygur people, of whom the incidence of ICC was 622/100,000 in 2008 [20], much higher than the incidence in the Han [2]. We speculated that TFPI2 methylation in different lesion grades was likely relative to ethnic differences. There have been few reports of the differences in the epigenetics of these populations. We analyzed the difference in TFPI2 methylation between the Uygur and Han cohorts across the four lesion classifications. Statistical significance was thus demonstrated only for CpG_31 of normal pathology samples and CpG_24.25.26 of CIN2/3 samples. We first employed a Student’s t-test, a rapid assay that allowed us to evaluate the differential methylation level of each CpG site amongst all Uygur and Han patients. The results suggest that there is a weak difference in TFPI2 methylation between Uygur and Han patients in the process of tumor formation. This weak difference may play a role in the ethnic disparity of cervical cancer incidence.

In order to elucidate the pathogenesis of cervical cancer, we analyzed the correlation of TFPI2 methylation between each CpG site and lesion grade in the Uygur and Han cohorts. This was further supported by a Spearman analysis, which revealed a stronger correlation between CpG site and lesion grade in Uygur patients than in Han patients. This may be particularly of importance in explaining the high incidence of cervical cancer in the Uygur people.

It is widely accepted that HPV is the main etiological factor determining cervical cancer development [21]. In the present study, we found no difference in HPV16 infection rates between the ICC patients of both cohorts. We also evaluated the difference between the four lesion grades in each cohort and found a significant difference in HPV16 infection rate across the four lesion grades in the Uygur cohort, further verifying the previous conclusion that HPV16 infection and its integration in host genome is a key event in the malignant transformation of cervical cells [22]. Earlier studies, both semi-quantitative [23] and quantitative [12, 24-29], also demonstrated a correlation between HPV viral load and disease progression. In some studies, however, a synergistic effect between HPV16 infection and other factors was not examined. We compared the presence of HPV16 infection and TFPI2 DNA methylation using a Student’s t-test. The results revealed that three CpG sites (CpG_6, CpG_15, and CpG_23) were differentially methylated in Uygur; however, there was no difference in the Han patients (Table 6). To our knowledge, this is the first report of an association between HPV infection and DNA methylation. Our data indicate a strong association between HPV16 infection in cervical cancer and TFPI2 DNA methylation, and we speculate that there is a synergistic effect of HPV16 infection and TFPI2 methylation in the progression of cervical cancer in Uygur but not Han patients.

Our results give insight into a potential mechanism responsible for the high incidence of cervical cancer in Uygur, while the reason for the differential morbidity associated with the disease amongst groups living in the same region in the northwest of China is not yet clear. Regarding breast cancer, Shan et al. [30] suggested that management strategies should be implemented to improve patient outcomes due to the differential characteristics of Uygur
breast cancer patients as compared to Han breast cancer patients, including their lower survival rates. Similarly, further research into whether different ethnic groups have varying treatment strategies regarding cervical cancer is also necessary.

Thus far, most studies on cancer have been performed in urban or high-income regions and few have examined low-income populations. It is worth paying more attention to the remote western China, especially the city-Kashi in western Xinjiang where Uygur is the main resident. In addition, the majority of Kashi residents have low incomes. More than 92% of low-income women lived on US$1.00 per day or less [31]. We should focus on the higher incidence of cervical cancer in this region, because whether the high incidence of cervical cancer in Xinjiang results from geographic, economic, or genetic/epigenetic factors - or a combination thereof - remains to be fully determined.

In conclusion, **TFPI2** hypermethylation may play an important role in the tumorigenesis of cervical cancer. Moreover, the difference in **TFPI2** methylation between Uygur and Han patients may contribute to high incidence of cervical cancer in Uygur and the disparity between the two groups. A synergistic effect of HPV16 infection and **TFPI2** methylation in the progression of cervical cancer in Uygur, but not Han, patients was also determined. These results are useful in understanding the etiology of cervical cancer in the Uygur people and will aid in future studies of Uygur women’s health.

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**Disclosure of conflict of interest**

None.

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TFPI2 methylation in cervical cancer


# TFPI2 methylation in cervical cancer

## Table S1. Comparison of TFPI2 methylation between the four lesion grades in the Uygur cohort

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<tbody>
<tr>
<td>F</td>
<td>3.495</td>
<td>1.697</td>
<td>2.121</td>
<td>1.981</td>
<td>4.583</td>
<td>1.946</td>
<td>6.645</td>
<td>3.063</td>
<td>2.413</td>
<td>2.968</td>
<td>3.256</td>
</tr>
<tr>
<td>P</td>
<td>0.019*</td>
<td>0.184</td>
<td>0.105</td>
<td>0.124</td>
<td>0.005**</td>
<td>0.133</td>
<td>&lt; 0.001***</td>
<td>0.034*</td>
<td>0.074</td>
<td>0.038*</td>
<td>0.027*</td>
</tr>
</tbody>
</table>

Note: Half of the CpG sites have different methylation levels between different lesion grades in Uygur cohort. Kruskal-Wallis H test was used to analyze the difference. *P < 0.05, **P < 0.01, ***P < 0.001.

## Table S2. Comparison of TFPI2 methylation between the four lesion grades in the Han cohort

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>4.645</td>
<td>2.708</td>
<td>1.13</td>
<td>2.46</td>
<td>1.575</td>
<td>1.895</td>
<td>3.304</td>
<td>4.704</td>
<td>3.081</td>
<td>2.298</td>
<td>3.899</td>
</tr>
<tr>
<td>P</td>
<td>0.004**</td>
<td>0.049*</td>
<td>0.34</td>
<td>0.067</td>
<td>0.2</td>
<td>0.135</td>
<td>0.347</td>
<td>0.023*</td>
<td>0.004*</td>
<td>0.030*</td>
<td>0.082</td>
</tr>
</tbody>
</table>

Note: There is a significant difference in TFPI2 methylation between the 4 lesion grades at some CpG sites. Kruskal-Wallis H test was used to analyze the difference. *P < 0.05, **P < 0.01.
**Table S3.** The difference between the four lesion grades of HPV16 infection in Uygur cohort (n = 123 cases)

<table>
<thead>
<tr>
<th>Lesion grades</th>
<th>HPV16 n (%)</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1 (4.8%)</td>
<td>20 (95.2%)</td>
<td>35.748</td>
</tr>
<tr>
<td>CIN1</td>
<td>3 (27.3%)</td>
<td>8 (72.7%)</td>
<td></td>
</tr>
<tr>
<td>CIN2/3</td>
<td>15 (41.7%)</td>
<td>21 (58.3%)</td>
<td></td>
</tr>
<tr>
<td>ICC</td>
<td>42 (76.4%)</td>
<td>13 (23.6%)</td>
<td></td>
</tr>
</tbody>
</table>

Note: There is a significant difference in HPV16 infection between the four lesion grades in Uygur cohort. "+" is HPV16-positive infection, "-" is HPV16-negative infection. Chi-square test was used in this analysis. $P$ values were obtained from comparisons between 4 lesion grades in detecting HPV16 infected in Uygur cohort. ***$P$ < 0.001.

**Table S4.** The difference in HPV16 infection between CIN2/3 and ICC in the Han cohort

<table>
<thead>
<tr>
<th>Lesion grades</th>
<th>HPV16 n (%)</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN2/3</td>
<td>24 (63.2%)</td>
<td>14 (36.8%)</td>
<td>0.338</td>
</tr>
<tr>
<td>ICC</td>
<td>23 (53.5%)</td>
<td>10 (46.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Note: There is not a significant difference in HPV16 infection between CIN2/3 and ICC in the Han cohort. Chi-square test was used in this analysis.