Comparative study of HPV16 integration in cervical lesions between ethnicities with high and low rates of infection with high-risk HPV and the correlation between integration rate and cervical neoplasia

LILI HAN[1,2], TUERXUNAYI MAIMAITIMING[2], SULAIYA HUSAIYIN[2], LIN WANG[2], KUNDUOZI WUSAINAHONG[2], CHUNHUA MA[2] and MAYINUER NIYAZI[2]

[1]Postgraduate College of Xinjiang Medical University, Urumqi, Xinjiang 830011; [2]Department of Gynecology, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi, Xinjiang 830054, P.R. China

Abstract. The etiology of a high incidence of cervical cancer in populations with a low human papillomavirus (HPV) infection rate is unclear. The current study aimed to investigate the role of HPV16 DNA integration in cervical lesions in women of Han and Uygur ethnicity and to explore the association between viral integration and a high cervical cancer morbidity with a low HPV infection rate. DNA was extracted from the biopsy specimens of cervical lesions of 379 patients of Uygur ethnicity and 464 patients of Han ethnicity, and multiple quantitative polymerase chain reaction (qPCR) assays were performed to determine the copy numbers of the HPV16 E2 and E6 genes. The copy number of the HPV16 DNA was evaluated according to the E2/E6 ratio. Among these cases, 122 Uygur and 121 Han specimens were found to be HPV16 positive. In the two populations, the percentage of cases with HPV16 integration (the sum of integrated-type infection only or a mixture of free-and integrated-type infection) increased with the grade of the cervical lesions (P<0.001). Within groups with the same cervical lesion grade, no significant differences in HPV16 integration were found between women of Uygur and Han ethnicity (rank sum test, P>0.05). No significant differences in the distribution of the HPV16 integration rate according to lesion grade were found in either population (P>0.05). When the two subpopulations were considered as one sample population, the integration rate significantly increased with lesion grade (P=0.02). These results indicate

that the integration rate of HPV16 E2 may serve as a molecular biological marker for the development of cervical lesions.

Introduction

Cervical cancer is the second most common malignant tumor in women throughout the world, ranking just behind breast cancer (1). The essential pathogenic factor in cervical cancer is the persistence of high-risk human papillomavirus (HPV), which is a double-stranded DNA virus with a capsid (2).

To date, numerous studies have reported on the integration of HPV DNA into the host genome in precancerous uterine cervical lesions and cervical cancers (3). High-risk HPV DNA integration into the host genome is a key factor eliciting malignant transformation in cervical lesions; both the HPV16 integration rate and the persistence of HPV16 infection demonstrate a positive correlation with the cervical lesion grade (4). Although the integration rates of HPV16 and HPV18 DNA increase with an increase in the malignancy level, the mixed type, that is, cases in which both free-type and integrated-type infection is present, is much more frequently observed in HPV16 integration. The integration of HPV16 and HPV18 into the host genome is an early step in cervical tumor development, and the incidence rate of HPV DNA integration increases with the aggravation of cervical lesions (5). HPV DNA integration has been detected in 7.4% of lesion tissues in HPV16-positive precancerous lesions of the uterine cervix, but integration occurs only in severe cervical lesions (6). These findings indicate that integration is an essential event in the progression of cervical lesions.

The Xinjiang Uygur autonomous region of China is an area with high morbidity from cervical cancer but a low HPV infection rate. The infection rate with high-risk HPV has been reported to be as low as 7.25% in women of Uygur ethnicity in Xinjiang Hotan Prefecture (7-9), compared with 12.1% in women of Han ethnicity (10). By contrast, the cervical cancer morbidity rate in women of Uygur ethnicity has been reported to be as high as 526 per 100,000 (11). The cause of the high morbidity but low HPV infection rate in Uygur women is

Correspondence to: Professor Mayinuer Niyazi, Department of Gynecology, People's Hospital of Xinjiang Uygur Autonomous Region, 91 Tianchi Road, Urumqi, Xinjiang 830054, P.R. China
E-mail: hanlili29@163.com

unclear, and the pathogenesis of HPV remains under investigation. Loss of the E2 gene and viral gene integration into the host genome elicits the progression of precancerous lesions from low grade to high grade, which increases the risk of cervical cancer (3,12,13). It may be questioned whether viral integration occurs at earlier stages in lesions in HPV16-infected women of Uygur ethnicity, whether the probability of integration is higher in Uygur women than in Han women with the same grade of lesion, and whether DNA fragments are more likely to break in women of Uygur ethnicity than in those of Han ethnicity. These questions motivated this comparative study.

Since the majority of previous studies have focused on the association of HPV16 integration with the development of cervical cancer (14-19), in the present study, HPV16-positive women were included as the subjects. A multiple quantitative polymerase chain reaction (qPCR) assay was performed to determine the copy numbers of HPV16 E2 and E6 in cervical cancer tissues and precancerous lesions. The physical state of the virus indicated by the E2/E6 ratio was then used to compare the HPV16 DNA integration rate in different grades of cervical lesions and to explore the correlation between integration and cervical lesion grade. Additionally, the HPV16 integration rates in patients of Uygur and Han ethnicity were compared to explore the role of HPV16 integration in different cervical lesion grades in women of different ethnicities.

Materials and methods

Patients and specimens. The specimens were biopsy or surgical specimens from 379 women of Uygur ethnicity and 464 women of Han ethnicity with cervical lesions treated in the Department of Gynecology of the People's Hospital of Xinjiang Uygur Autonomous Region from August 2010 to March 2011. The specimens were reviewed by two pathologists with high academic positions. No radiotherapy or chemotherapy was administered prior to biopsy or surgery. Pregnancy, immunologic insufficiency and cervical surgical history were the exclusion criteria. The specimens were stored at -80°C prior to analysis. The ages of the patients of Uygur ethnicity ranged from 24 to 67 years, with a mean of 50±12-years. The patients of Han ethnicity ranged in age from 24 to 81-years, and had a mean age of 46±14-years. The lesion types of the patients' specimens are presented in Table I.

Approval for this study was obtained from the Institutional Review Board of the People's Hospital of Xinjiang Uygur Autonomous Region (Xinjiang, China). All participants provided written informed consent.

According to Wu *et al* (20), cervical intraepithelial neoplasia (CIN) is pathologically characterized by cervical dysplasia and carcinoma *in situ* with nuclear heterogeneity such as large, deeply stained nucleus with different sizes and appearance and maldistributed chromatins. Based on the degrees of cell changes and the scales of heterotypical cells, the following grades may be classified: 1, Mild atypical hyperplasia (CINI), in which cells show a mild degree of heterogeneity in an untidy arrangement but with polarity and the abnormally proliferated cells are confined to the lower 1/3 part of the epithelial layer; 2, moderate atypical hyperplasia (CINII), in which cells show noticeable heteromorphism in a disordered arrangement and the abnormally proliferated cells

occupy the lower 2/3 part of the epithelial layer; and 3, severe atypical hyperplasia and carcinoma *in situ* (CINIII), in which cells show noticeable heteromorphism without polarity and the abnormally proliferated cells occupy the lower 2/3 part of the epithelial layer or even expand to the whole layer. Squamous cell carcinoma (SCC) refers to the condition in which the moistening interstitium of the focus exceeds the carcinoma *in situ*, showing mesh- or mass-like confluent invasion, and the pathological characteristics of cervicitis (N) may include cervical polyp, cervical hypertrophy and Nabothian cysts.

Reagents. The TIANamp Genomic DNA kit for genomic DNA extraction from blood, cells and tissue (spin column type) was purchased from Tiangen Biotech (Beijing, China). Primers and probes for HPV16 E2 and HPV16 E6 were synthesized by Takara Biotechnology (Dalian, China) and are listed in Table II. The real-time PCR SuperMix kit, containing Premix Ex Taq™ and ROX reference dye, was purchased from Takara Biotechnology. The pCR®-XL-TOPO® 3.5 kb HPV16 plasmid and SiHa cell line DNA were gifts from Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, Xinjiang University (Xinjiang, China).

Extraction of the sample DNA. DNA was extracted from the specimens according to the instructions provided by the manufacturer of the TIANamp Genomic DNA kit for genomic DNA extraction from blood, cells and tissue. Clinicopathological detection was performed with a nucleic acid spectrophotometer (Beckman DU 700; Beckman Coulter, Inc., Brea, CA, USA).

Preparation of standard samples. The copy number was determined using the following formula: Copy number = weight (g) x $6.23x10^{23}$/324.5 x 2 x length of plasmid DNA (where $6.23x10^{23}$ represents the molecular number of 1 mol of substance and 324.5 is the average molecular weight of a basic group). The copy number of the pCR®-XL-TOPO® 3.5 kb HPV16 plasmid was determined to be $4.8x10^{10}$ copies/μl, and the plasmid was then stored at -20°C. When adding the samples to the PCR mixes, the concentration of the plasmid was diluted to $4.8x10^{10}$, $4.8x10^{9}$, $4.8x10^{8}$, $4.8x10^{7}$, $4.8x10^{6}$, $4.8x10^{5}$, $4.8x10^{4}$ and $4.8x10^{3}$ copies/μl to serve as standard samples.

Determination of the E2 and E6 copy number through multiple qPCR. The mixes for qPCR were prepared with the following reagents (total volume, 30 μl): 15 μl ddH$_2$O, 9 μl Premix Ex Taq™ (2X), 0.6 μl ROX reference dye (50X), 0.6 μl HPV16 E2 forward primer (10 μM), 0.6 μl HPV16 E2 reverse primer (10 μM), 0.6 μl HPV16 E6 forward primer (10 μM), 0.6 μl HPV16 E6 reverse primer (10 μM), 0.5 μl HPV16E2 Taq Man probe (20 μM), 0.5 μl HPV16E6 Taq Man probe (20 μM), and 2 μl template DNA. The concentration of each standard DNA sample was measured three times, and the concentrations of the specimen DNA samples were measured twice. DNA extracted from SiHa cells was used as the negative sample control, the prepared standard sample of $4.8x10^{7}$ copies/μl was used as the positive system control, and a DNA blank was used as the negative system control. The PCR amplification was performed in an ABI 7500 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA) with the following reaction conditions: Initial denaturation at 95°C for 30 sec followed

Table I. Lesion types of the specimens from women of Uygur and Han ethnicity.

Ethnicity	Inflammation	CIN I	CIN II	CIN III	Cervical cancer	Total
Uygur	190	96	12	30	51	379
Han	250	113	14	50	37	464

CIN, cervical intraepithelial neoplasia.

Table II. Primers and probes for quantitative polymerase chain reaction.

Amplified fragments	Primers/probes	Sequence (5'→3')	Size (bp)
HPV16 E2 (nt 3452-3590)	Forward primer	GAAACACAGACGACTATCCA	195
	Reverse primer	TCCGTCCTTTGTGTGAGCTGT	
	Probe	HEX-CCAAGACAGAGCCAGACAC-Eclipse	
HPV16 E6 (nt 188-382)	Forward primer	GAATGTGTGTACTGCAAGCA	132
	Reverse primer	GTTGTATTGCTGTTCTAATGTTGT	
	Probe	FAM-CAGCATATGGATTCCCATCTC-Eclipse	

HPV, human papillomavirus; nt, nucleotide.

Table III. Case population and rate of HPV16-positive cervical lesions according to lesion type in women of Uygur and Han ethnicity [n, (%)].

Ethnicity	Inflammation	CIN I	CIN II	CIN III	Cervical cancer	Total
Uygur	20 (10.5)	32 (33.3)	6 (50.0)	20 (66.7)	44 (86.3)	122
Han	22 (8.8)	34 (30.1)	7 (50.0)	28 (56.0)	30 (81.1)	121

HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia. χ^2=4.211, P=0.3782.

by PCR at 95°C for 5 sec, 58°C for 34 sec, and 72°C for 30 sec (for 40 cycles).

Statistical analysis. Samples with HPV16 E6 copy numbers >0 were considered HPV16 positive. The numbers of cases with cervical lesions in the different populations were expressed as an absolute number and percentages, and the HPV16 integration rates were expressed as the mean ± standard deviation. The data were processed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The HPV16-positive rates of the different populations were compared using the χ^2 test, the HPV16 integration states in the various cervical lesion grades of the different populations were compared with the Kruskal-Wallis H test, and the integration states and integration ratios within the same cervical lesion grade in the different populations were compared using the Wilcoxon test. Differences of P<0.05 were considered statistically significant, with a test power of 0.8.

Results

Clinicopathological information regarding HPV16-positive patients. Detection with a nucleic acid spectrophotometer indicated that the extracted DNA had a concentration >150 ng/μl.

The results from the double PCR detection of HPV16 DNA in the patients are listed in Table III. The Uygur group and the Han group did not show any differences in the grades of the cervical lesions.

Standard curves. Standard curves were established based on the multiple qPCR data for E2 and E6 in the standard samples (Fig. 1). The equations used to establish the standard curves for E2 and E6 were: Ct = -3.34$\log X_0$ + 40.461 and Ct = -3.417$\log X_0$ + 40.521, where X_0 is the original copy number and Ct represents the cycle threshold reflecting the number of cycles required for the fluorescence intensity in the tube to reach the designated threshold. Based on the Ct values for each tube, the E2 and E6 copy numbers were calculated, and 20 E2/E6 ratios were obtained. Of note, the E6 gene was not detected in one low-concentration standard sample. The 95% confidence interval for the 20 E2/E6 ratios was calculated to be 0.81-1.20. The critical value of 0.81 was confirmed as the demarcation between HPV16 free-type infections and integrated-type infections. Specifically, E2/E6=0 indicated an integrated-type infection, E2/E6>0.81 indicated a free-type infection, and 0<E2/E6<0.81 indicated a mixed-type infection (both free-type and integrated-type).

Table IV. Comparison of the integration states of HPV16 [n, (%)].

Lesion grade	Uygur (n=122)			Han (n=121)			Z^a	P-value
	Free	Mixed	Integrated	Free	Mixed	Integrated		
N	16 (13.11)	4 (3.28)	0 (0)	15 (12.40)	7 (5.79)	0 (0)	-0.86	0.39
CIN I	19 (15.57)	13 (10.66)	0 (0)	17 (14.05)	17 (14.05)	0 (0)	-0.76	0.45
CIN II/III	8 (6.56)	17 (13.93)	1 (0.82)	9 (7.44)	25 (20.66)	1 (0.83)	-0.34	0.73
SCC	9 (7.36)	32 (26.23)	3 (2.46)	7 (5.79)	21 (17.36)	2 (1.65)	-0.26	0.80
χ^{2b}	26.18			16.14				
P-value	0.00			0.00				

^{a}Wilcoxon test; ^{b}Kruskal-Wallis H test. HPV, human papillomavirus; N, negative; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.
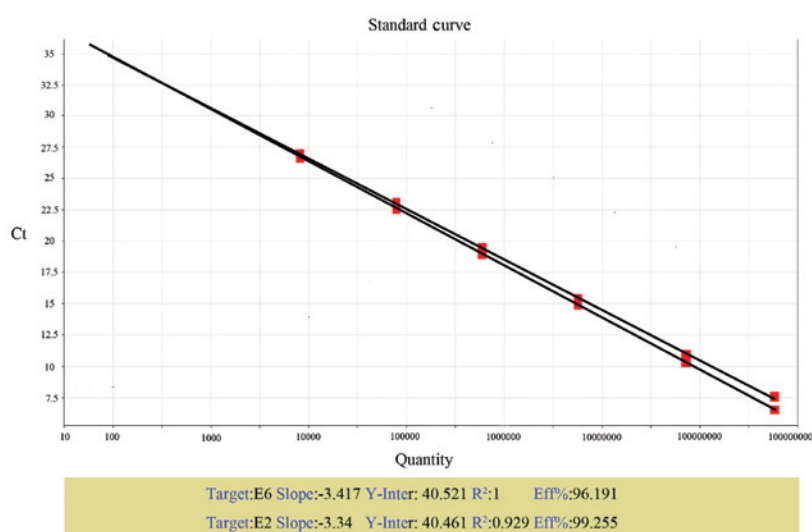

Figure 1. Standard curve for the standard samples based on multiple quantitative polymerase chain reaction results.

HPV16 integration rates in different grades of cervical lesions. The HPV16 integration ratios in cervical lesions of different grades were detected. In the positive system control, the ratio of E2/E6 was 0.96. E2 and E6 were not detected in the negative system control. In the negative sample control, the ratio of E2/E6 was 0. The integration state of HPV16 in the patients of Uygur and Han ethnicity is shown according to lesion grade (N, CIN I, CIN II/III or SCC) in Table IV. According to the Kruskal-Wallis H test for multi-sample comparisons, the type distribution within the same population showed a significant difference (P<0.05); specifically, as the lesion grade increased, the proportion of free-type HPV16 infections decreased, and the proportion of integrated-type infections increased. Between the Uygur and Han populations, the proportions of integration showed no significant differences within the same cervical lesion grade (P>0.05).

HPV16 integration rates within different cervical lesion grades in patients of Uygur and Han ethnicities. The integration rate was defined as 1-E2/E6. The HPV16 integration rates for the lesions of grades N, CIN I, CIN II, CIN III and SCC are shown in Figs. 2 and 3. In the Uygur patients with lesion grades N and CIN I and in the Han patients with lesion grades N, CIN I and CIN II, the integration rates were <0.5. In the lesions of grades N, CIN I, CIN II, CIN III and SCC in patients of either Uygur or Han ethnicity, the integration rate of HPV16 appeared to increase as the grade of the cervical lesions increased; however, the increase was not found to be statistically significant according to the Kruskal-Wallis H test (Uygur: χ^2=2.44, P=0.30; Han: χ^2=4.12, P=0.13).

Between the Uygur and Han patients, there was no significant difference in the HPV16 integration rate within the same grade of lesion (Wilcoxon test of 2 independent samples, P>0.05; Table V). Irrespective of the differences between the two populations, the Uygur and Han patients were considered as one sample to increase the sample size. As the grade of the lesions increased from N to CIN I, CIN II/III and SCC, the integration rate increased gradually, and the increase was statistically significant (Kruskal-Wallis H, χ^2=8.42, P=0.02).

Discussion

In this study, the proportion of integration within the same cervical lesion grade demonstrated no statistically significant
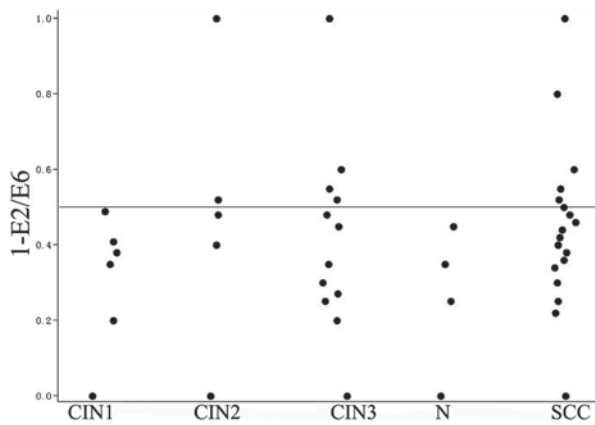
Figure 2. Distribution of the HPV16 integration rates according to cervical lesion grade in Uygur patients. HPV, human papillomavirus; CIN, cervical intraepithalial neoplasia; N, negative; SCC, squamous cell carcinoma.
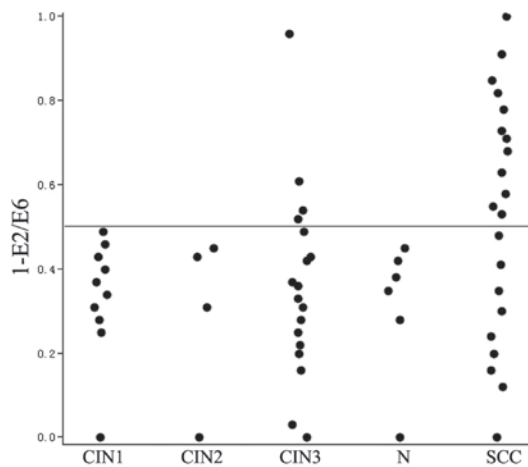

Figure 3. Distribution of the HPV16 integration rates according to cervical lesion grade in Han patients. HPV, human papillomavirus; CIN, cervical intraepithalial neoplasia; N, negative; SCC, squamous cell carcinoma.

Table V. Comparison of the HPV16 integration rates between the patients of Uygur and Han ethnicity with the same cervical lesion grades (mean ± standard deviation).

Grade	Uygur	Han	Z[a]	P-value
N	0.16±0.12	0.13±0.10	-0.57	0.60
CIN I	0.27±0.18	0.23±0.15	-0.54	0.59
CIN II/III	0.38±0.26	0.32±0.22	-0.58	0.56
SCC	0.41±0.24	0.51±0.30	-0.56	0.58

[a]Wilcoxon test. HPV, human papillomavirus; CIN, cervical intraepithalial neoplasia; N, negative; SCC, squamous cell carcinoma.

difference between Uygur and Han ethnicities. Even in the group of patients with CIN II/III grade lesions, the integration ratio in the women of Han ethnicity appeared to be higher than that in the women of Uygur ethnicity. Neither of these findings is able to account for the high morbidity but low HPV infection rate observed in the Xinjiang Uygur population. Based on the results of this study, there are four potential explanations. First, E2 contains an N terminus, C terminus and hinge region. In the study by Zhang *et al*, the miss rates for the N terminus, hinge region and C terminus were 71.88, 50.00 and 21.88%, respectively (21). It may be possible that frequent fractures occur outside of the hinge region in women of Uygur and Han ethnicity in Xinjiang. This possibility is dependent on the complete detection and analysis of the fracture sites of E2, and even of E1, to identify whether actual differences in HPV16 integration rates exist between Uygur and Han women. Second, the small sample size limited the detection accuracy; thus, increasing the sample size might improve the detection power in further studies. Third, no consensus has been reached regarding the pathogenesis of HPV16 integration into the host genome during cervical cancer development. Among patients with CIN I, CIN II/III and SCC from France, the HPV16 integration rates were 64, 83.3 and

82%, respectively. Although the integration rate increased as the grade of the lesion increased, this change was not statistically significant, which might be attributable to the small sample size (22). Another previous study of CIN and cervical cancer cases revealed that the average HPV16 integration rate was 68%, and the researchers concluded that HPV16 integration is not an essential pathogenic factor in the development of cervical cancer (23). In addition, a previous study demonstrated different proportions of HPV16 integration within the same cervical lesion grade from different areas in the world (20). Finally, in women with cervical cancer, multiple infections occur more often in Uygur women than in Han women. As a carcinogenic mechanism, multiple infections might facilitate amplification of the telomerase RNA component (*TERC*) gene, resulting in the higher morbidity of cervical cancer in Uygur women than in Han women (24). Certainly, certain customs and conditions of the Uygur population, including early marriage, early pregnancy, multiple births, poor health habits, inconvenient transportation, insufficient medical facilities and lack of a cervical cancer screening system, may also substantially contribute to the high morbidity of cervical cancer.

The proportion of HPV16 integration can provide a cross-sectional perspective of the integration incidence in a population and might reflect the difficulty of integration in different cervical lesion grades. The integration rate can also provide a longitudinal perspective of integration and may indicate the intensity of viral integration in individuals. This latter aspect might be beneficial for disease assessments of individual patients (25) and could trigger shunting effects that affect the prognosis of HPV-positive CIN II cases (26). The results of the present study demonstrate different degrees of HPV16 integration between different grades of CIN and cervical cancer in the study population. In the N, CIN I, CIN II/III and SCC groups, the proportion of free HPV16 decreased as the lesion grade increased, but the proportion of integration (mixed- and fractured-type) increased gradually. The integrated type was found in CIN II/III and SCC lesions, and the percentage of the integrated type was highest in SCC. Specifically, as the grade of the cervical lesion increased, a larger rate of HPV16 integration was observed, which revealed that HPV16 integration into the host genome is a key pathogenic factor in cervical cancer. This finding is consistent with studies of women of Han ethnicity in Sichuan and Hubei conducted by Zheng *et al* (27), Shi *et al* (28) and Li *et al* (29).

The mean integration rates in the N, CIN I, CIN II/III and SCC groups demonstrated no statistically significant differences between the Uygur and Han patients. When the differences between the populations were ignored and the Uygur and Han women were considered as one sample, it was found that the integration rate increased as the lesion grade increased from N to CIN I, CIN II/III and SCC to a statistically significant degree. This finding indicates that an increased sample size might help to elucidate the association between viral integration and the progression of cervical lesions. Once the association between increasing viral integration and cervical lesion progression is established, the comparison between Uygur and Han ethnicities will become significant.

A previous study detected HPV16 in cases graded as N, CIN I, CIN II/III and SCC with median HPV16 E2/E6 ratios of 0.75, 0.58, 0.51 and 0.36, respectively. The ratio tended to decrease with increasing cervical lesion grade (22). That investigation revealed that viral loads >22,000 copies/1,000 cells and yielding an E2/E6 ratio <0.5 tend to progress into higher grade lesions. This finding may guide further studies concerning the integration rate in Han women using larger sample populations.

In conclusion, HPV16 integration was found to be an essential factor contributing to the progression of cervical lesions in women of Uygur and Han ethnicity, and no differences in HPV integration were observed between the two populations. In addition, no correlation was observed between the integration rate and the progression of the cervical lesion grade. In further studies, larger sample sizes and more sensitive detection methods are required to explore the objective pathogenesis of the high cervical cancer morbidity in women of Uygur ethnicity.

Acknowledgements

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