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Application of molecular genotyping to determine prevalence of HPV strains in Pap smears of Kazakhstan women

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ABSTRACT

Objectives: Human papillomavirus is the main causative agent for cervical cancer. However, few data are available about HPV prevalence in Kazakhstan. The aims of this study were to genotype HPV DNA in Pap smear samples of women to determine prevalence of carcinogenic HPV types in Astana, Kazakhstan and to analyze the association between HPV positivity and the cytology results of patient samples.
Methods: Pap smear materials were obtained from 140 patients aged 18-59, who visited the outpatient gynecological clinic. Microscopic examination was done to detect dysplasia, and HPV genotyping was done using real-time multiplex PCR.
Results: HPV testing showed that among 61 HPV positive patients, the most prevalent types were 16 and 18. Microscopic examination showed that 79% of the samples had normal cytology, while 13% had CIN grade I, 5% had CIN grade II, and 3% had CIN grade III. The analysis revealed that 12% of the samples had CIN cytology and presence of HPV. Approximately 31% had HPV without cervical dysplasia, while 8% of samples were CIN positive without HPV infection. A statistically significant relationship between HPV 16 and HPV 33 positive samples and CIN grade II and III was found.
Conclusions: Overall, this study will help to strengthen and guide health policy implementation of primary and secondary cervical cancer prevention strategies in Kazakhstan.

1. Background

Cervical cancer is the third most diagnosed type of cancer and the fourth leading cause of death in women worldwide[1]. Approximately 99% of all cervical cancer cases have been linked to Human papillomavirus (HPV) infection, which has also been associated with other anogenital cancers (anus, vulva, vagina and penis), and head and neck cancers[2]. HPV is a small non enveloped double-stranded DNA virus of Papillomaviridae family[3]. Of the more than 100 HPV types, 15 (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) were identified as high risk (HR) due to their strong correlation to cancers of the anogenital region[4]. HPV types 16 and 18 are responsible for about 70% of all cervical cancer cases worldwide, while other HPV types like 31, 33, 35, 45, 52 and 58 account for 20% of cervical cancers in the world[5]. Screening for cervical cancer with the Papanicolaou cytology technique, or Pap smear test for short, has become widely accepted. The Pap smear test is aimed to detect cervical intraepithelial neoplasia (CIN), which is a premalignant transformation in the cervix. Additionally, HPV DNA testing for HR-HPV types is also recommended to improve the accuracy of screening for cervical cancer, and to increase chances for recovery[6].

Current estimates for Kazakhstan indicate that every year 2,789 women are diagnosed with cervical cancer and 982 die from the disease[2]. Cervical cancer in Kazakhstan ranks as the second most frequent cancer among women and the first most frequent cancer among women between 15 and 44 years of age with the incidence of 32.8[2]. However, there is still lack of sufficient information about the prevalence of HR-HPV types in Kazakhstan. Having more information about the types of HPV strains that are prevalent in patients with abnormal cytology and hence are at high risk for developing cervical cancer can provide guidance regarding preventative measures against cervical cancer. This pilot study

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on HPV in Kazakhstan was undertaken with two main objectives; the primary objective was to determine the most prevalent HPV types in Astana, Kazakhstan by genotyping HPV DNA in Pap smear samples of women that are at risk to develop cervical cancer. The second objective of this study was to analyze the association between HPV positivity and the cytology results of patient samples. This also serves as a feasibility study that will form the basis for a larger study. Given the high incidence of cervical and other HPV associated cancers in Kazakhstan and the Central Asian countries, findings from such studies could contribute toward informing relevant policies related to screening and preventative vaccination and other public health HPV programs in the region.

2. Methods

2.1. Study population and sample collection

The total number of patients in this study was 140 women of ages 18 – 59 years. The samples were collected as part of a screening procedure, called Pap smear, from all patients who attended the gynaecologist's office at University Medical Center (UMC), Astana, in the period from December, 2015 to April, 2016. The samples were collected in two sets in order to do microscopic analysis for abnormal cytology and for HPV DNA genotyping. The sample collection and analysis (microscopy and RT-PCR) were done on the same day.

2.2. Ethical consideration

The Nazarbayev University Ethical Committee (IREC) granted exemption from IREC review for this study. Patient confidentiality was maintained whereby specific patient information (including name, contact and other sensitive personal information) was only accessible to the clinicians, and samples were number-letter coded to the investigators involved to conduct the protocols proposed for the study. Patient confidentiality was maintained throughout, and after completion of the study.

2.3. HPV DNA genotyping and cytological examination of samples

For HPV genotyping, real time multiplex PCR methodology using the specific PCR kit were used, following manufacturer's instructions (https://vector-best.ru/en/publ/list/maket_hpv.pdf - www.vector-best.ru). The laboratory method is based on simultaneous real-time multiplex PCR of HPV-specific DNA fragments and a noncompetitive internal control. The real time PCR instrumentation used for the assay is the CFX 96 Real -Time PCR (BIO-RAD). Microscopic examination of Pap smear samples was also performed using standard UMC hospital protocols to reveal cytological abnormalities[7].

2.4. Statistical analysis

All statistical analysis for this study was done with STATA 13.0 software. Simple statistical analysis such as determination of means, standard deviation, p-value, and odds ratio, was done for descriptive purposes. The association between age, HPV positivity status, and cytological pathology was analyzed with logistic regression.

3. Results

3.1. Prevalence of HPV types among positive samples

Twelve HPVs, which are HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59, genotypes were detected in our study samples using real-time PCR. Out of 140 samples analyzed by HPV genotyping, HPV DNA could be detected in 61 of samples (43.6% of total), and all 12 HPV genotypes could be identified collectively among these HPV positive samples. HPV DNA genotyping with real-time PCR showed that among 61 HPV positive samples, the most prevalent types detected were HPV 16 (18.4%) and HPV 18 (9.22%), followed by HPV types 33, 51 and 52 (nearly 5% each) (Figure 1). HPV types 59, 39, 31, 45, and 58 were found in at least 2% of the total amount of samples, while only about 1% or less of positive samples had HPV types 35 and 56.

3.2. Variation of cytology results among different age groups

The results of cytological examination and of HPV DNA genotyping with age distribution of patients are shown in Table 1. The age range of patients was from 18 to 59 years old, with a mean age of 33.5 ± 9.51 years (95% confidence interval (CI) 31.9 – 35.1 years). Among 140 cytology samples, 28 were identified as CIN grade 1, 2 or 3. The age group of 26-36 years had the highest number of HPV and CIN positive patients (Table 1). Regression analysis did not show statistically significant association between age and HPV positive status (p = 0.290). The results of Pap smear test

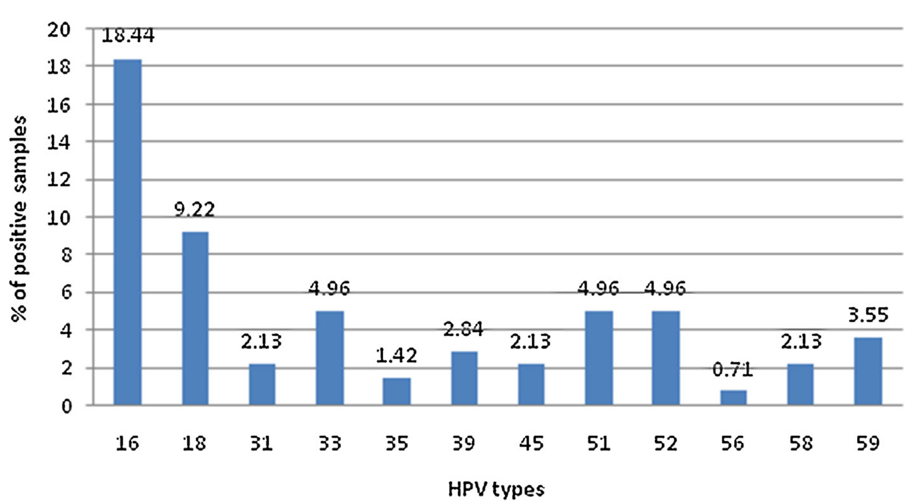

Figure 1. Distribution of HPV types among 61 HR-HPV positive patients, tested in Astana, Kazakhstan
Abbreviations
HPV = Human papillomavirus; HR-HPV = high risk HPV types.

Table 1
Age distribution among 140 patients with HPV and CIN

Age (years)	N women	HPV positive		CIN positive		OR	95% CI
		N	%	N	%*		
18-25	23	8	34.7	2	8.70	0.5	0.045 –5.51
26-36	76	38	50.0	17	2.24	1.25	0.27 – 6.5
37-47	25	9	36.0	5	20.0	1.37	0.14 – 14.5
48-59	16	6	37.5	4	25.0	1.33	0.10 – 18.0
Total	140	61	43.6	28	20.0		

Abbreviations: HPV = Human papillomavirus; CIN = Cervical intraepithelial neoplasia; OR = odds ratio; CI = confidence interval; * - percentage in this age group

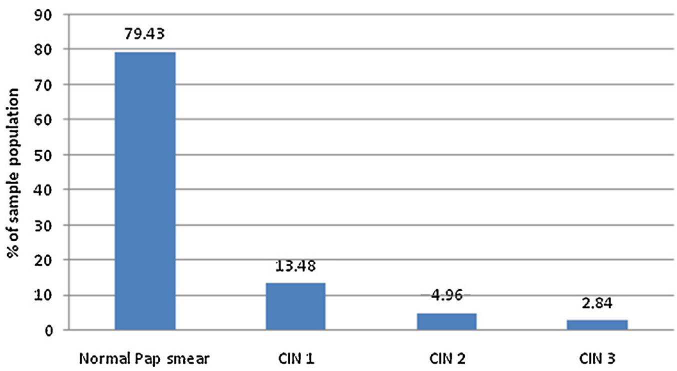

Figure 2. Representation of cytological examination among 140 tested women
Abbreviations
CIN = Cervical intraepithelial neoplasia.

showed that 13.5% of the 140 patients had grade 1 CIN, approximately 5% had CIN 2, and nearly 3% had grade 3 CIN, while 79% of samples did not have abnormal cytology (Figure 2).

3.3. Correlation between HPV and CIN in positive samples

The proportion of patients who were positive for both HPV and CIN was 12% (Figure 3) with the age range from 23 to 42 years. Patients who had only CIN without HPV infection constitute 8% of all patients, and the age range of CIN only positive patients was from 30 to 59 years. One third of the patients (age 18-57 years) had positive HPV test results but normal cytology from Pap smear. The remaining samples were negative for CIN and HPV. Logistic regression analysis showed statistically significant association between HPV types 16 and 33 and CIN grades 2 and 3 (Table 2).

4. Discussion

Our findings demonstrate that the most prevalent types of HR-HPV among 61 patients (43.6%) who appeared to be HPV positive,

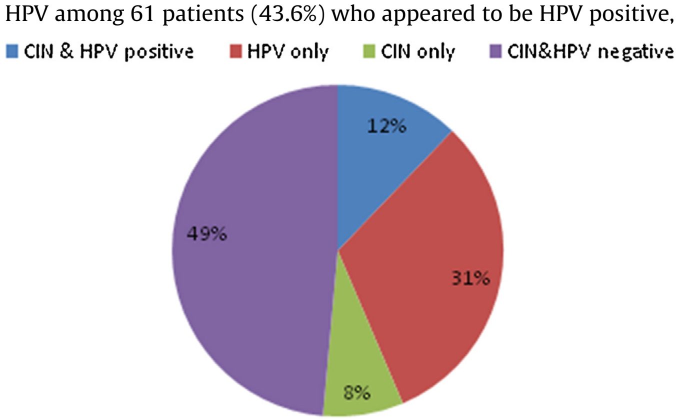

Figure 3. Predominance of HPV and CIN among 140 women tested at UMC, Astana, Kazakhstan.

Table 2
Association between HPV type and grade dysplasia

HPV type	Characteristic associated	P value	95% CI
16	CIN 2	0.043	1.08 - 137
	CIN 3	<0.005	$2.48x10^5$ – $9.72x10^7$
33	CIN 2	<0.005	8.52 - 1686
	CIN 3	<0.005	$9.81x10^5$ – $5.34x10^8$

Abbreviations: HPV = Human papillomavirus; CIN = Cervical intraepithelial neoplasia; OR = odds ratio; CI = confidence interval

are HPV 16 (18.4%) and 18 (9.22%), followed by HPV 33, 51, and 52. It is common practice to do both genotyping and microscopic examination in cervical cancer screening both in Western countries and in many Central Asian countries. However, from Kazakhstan, only one study has been published regarding the analysis of both genotyping and Pap smear samples from patients. That study detected total HPV high-risk infection in 26% of the population tested, with the dominating type being HPV 16 (10.7%)[8]. The prevalence of HPV 18 and 33 reported by this study was much smaller (3.61% and 1.25% respectively), though with their much larger sample size, this finding may be a more accurate reflection of prevalence for this region of Kazakhstan. Furthermore, epidemiologic studies worldwide show that HR-HPV types 16 and 18 are predominantly found in cervical cancer cases as well as in low- and high- grade cervical lesions (CIN 1, 2, 3)[9–11]. In addition to total prevalence of HR-HPV types, association between age and HPV positive status was analysed because some studies showed that the peak of HPV positivity usually occurs in women under age 35[6,12,13]. Although in our study we were able to observe that the majority of HPV positive patients were in the age range from 26 to 36, we did not find statistically significant correlation (with a p-value = 0.290) between age and having HPV infection. Most probably this can be explained by the fact that the sample size of 140 patients in our study was too small since an estimated minimum sample size of 189 is required in order to obtain the power to equal 0.8.

Fully supported by the government of Kazakhstan, the Pap smear test is recommended for women of reproductive age at 5-year intervals as a screening procedure for early detection of preventable cancers[14]. The results obtained in this study showed that 20% of tested women had CIN of grades 1 (13.5%), 2 (4.96%), and 3 (2.84%). After evaluation of the data we could see that 12% of participants had both cervical dysplasia and HPV infection. However, it is known that the Pap smear test has quite high false-negatives rate[15], which suggests that some of the HPV positive patients (31%) with normal cytology should be monitored for a certain time period. Monitoring HPV positive patients is necessary to see if the infection will be cleared by immunity in a 1-2 year period[16], or to detect abnormal cell proliferation at early stages and to start treatment. Clearance of the infection probably can also explain the fact that 8% of the patients in this study had dysplasia but not HPV infection. Statistical analysis showed that there is a statistically significant association between having HPV type 16 and CIN2 and CIN3, as well as being HPV type 33 positive and having CIN2 and 3 (Table 2). In these cases, the progression to a higher-grade neoplasia can be rationalized not only by the presence of HR-HPV infection, but also by persistence of the infection for a longer period of time. Persistence of the infection indicates longer exposure to carcinogenic proteins of HPV such as E6/E7, thus, transformation of normal basal cells to a higher grade of cervical neoplasia as well as to invasive cervical cancer[17,18].

There are several limitations in this study. First, because of constraints on patient availability, the sample size was smaller than the estimated number of participants required for obtaining the results with the power of 0.8. Second, not all samples selected for HPV DNA genotyping had abnormal cytology, which is a significant barrier for finding true association between the HPV type and cervical dysplasia. Third, the complete demographic data and clinical records of all patients were not available due to the unwillingness of some patients to provide personal information that may be relevant to the study. The suggestion for future studies is to increase the sample size and to perform the study in different parts of the country. Another option is to perform pooled analysis from the whole country as was done in a 2012 study in China and Europe where the prevalence of HR-HPV and CIN was determined with meta-analysis[18,19]. One significant finding from our study is that HPV DNA could be detected in Pap smear normal samples; this suggests that the HPV genotyping assay can be a valuable addition to the Pap smear test as effective and more thorough screening methodologies. Interventions provided to these early infections could potentially prevent progression to HPV-related cancers.

5. Conclusion

Overall, this pilot study was important not only to obtain more data regarding HPV prevalence among Kazakhstan women and to analyze the correlation between certain HPV types and cervical dysplasia, but also to provide the basis for future studies. In conclusion, having a well-informed understanding of the association of HPV infection and associated cancer could enhance the public's acceptance of screening and intervention programs to reduce morbidity and mortality due to HPV infection. Specifically, having a definitive knowledge regarding which strain is prevalent in Kazakhstan will provide guidance as to the best and most cost-effective choice of vaccine for protection of the population against HPV. The overall goal is to work toward informing public policy in terms of best practices that should be adopted with regard to screening and vaccination preventative programs that would result in significant reduction in the incidence and prevalence of cervical and other cancer types associated with HPV infection.

Ethics statement

The Nazarbayev University Ethical Committee (IREC) granted exemption from IREC review for this study. Patient confidentiality was maintained throughout, and after completion of the study.

Competing interests

The authors state no competing interests.

Authors' contributions

LN compiled, analyzed data and carried the statistical analyses, and prepared the manuscript. GA, NS and AA conceived the study, reviewed the data, provided feedback for figures, and edited the manuscript. NS provided clinical and intellectual assistance with regards to pap smear sample collection and provision for HPV genotyping. ZA and KS provided technical and intellectual assistance regarding HPV genotyping. SA provided intellectual input to contribute towards manuscript preparation. All authors reviewed and approved the final manuscript.

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