Prevalence of human papillomavirus in Mazandaran province, Islamic Republic of Iran

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انتشار فيروسة الورم الحليمي البشري في منطقة مازندران في جمهورية إيران الإسلامية

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الخلاصة: يقدّم هذا البحث تقريراً عن معدل انتشار أنواع فيروس الورم الحليمي البشري في 100 عينة من خزعات عنق الرحم في منطقة مازندران. وقد اكتُشِفَ دنا DNA فيروس الورم الحليمي البشري في 78.6٪ من حالات سرطانة عنق الرحم، وفي 64.3٪ من حالات خلل التنسج والحؤول، وفي 9٪ من الحالات الطبيعية. وقد وُجِدَ ارتباط يُعتدُّ به بين وجود دنا فيروس الورم الحليمي البشري وبين نشوء سرطانة عنق الرحم. واكتُشِفَ نوعا فيروس الورم الحليمي البشري 16 و18 في 60.6٪ من الحالات الإيجابية لسرطانة عنق الرحم، بينما وجد نوعا فيروس الورم الحليمي البشري 31 و33 في 21.2٪ من الحالات، والنوعان 6 و11 في 18.2٪ من الحالات. ومن بين حالات خلل التنسج والحؤول الإيجابية لفيروس الورم الحليمي البشري، كان 55.6٪ منها إيجابياً لفيروس الورم الحليمي البشري من النوعين 16 و18، وكان 22.3٪ منها إيجابياً لفيروس الورم الحليمي البشري من النوعين 6 و11 ، وكان 11.1٪ منها إيجابياً لفيروس الورم الحليمي البشري من النوعين 31 و33. بينما اكتُشِفَ فيروس الورم الحليمي البشري من النوعين 6 و11 في أربع عينات (أي 100٪) من الخزعات الطبيعية.

ABSTRACT We report the prevalence of human papillomavirus (HPV) types in 100 cervical biopsy specimens in Mazandaran province. HPV DNA was detected in 78.6% of cervical carcinoma cases, 64.3% of dys/metaplasia and 9% of normal cases. Significant correlation was found between the presence of HPV DNA and development of cervical carcinoma. HPV types 16 and 18 were detected in 60.6% of HPV-positive cervical carcinoma cases, whereas HPV31 and 33 were found in 21.2%, and HPV6 and 11 in 18.2%. Among HPV-positive dys/metaplasia cases, 55.6% were positive for HPV16 and 18, 22.3% for HPV6 and 11, and 11.1% for HPV31 and 33. Only HPV6 and 11 were detected in 4 (100%) normal biopsy specimens.

Prévalence du papillomavirus humain dans la province de Mazandaran (République islamique d'Iran)

RESUME Nous rapportons la prévalence des types de papillomavirus humain (PVH) dans 100 prélèvements biopsiques du col de l'utérus dans la province de Mazandaran. L'ADN du papillomavirus humain a été détecté dans 78,6 % des cas de carcinome du col de l'utérus, 64,3 % de dysplasies/métaplasies et chez 9 % des sujets ayant un col de l'utérus normal. On a trouvé une corrélation significative entre la présence d'ADN du papillomavirus humain et le développement d'un carcinome du col de l'utérus. Les types 16 et 18 de PVH ont été dépistés dans 60,6 % des cas de carcinome du col de l'utérus positifs pour le papillomavirus humain, tandis que les types 31 et 33 de PVH ont été trouvés dans 21,2 % des cas et les types 6 et 11 de PVH dans 18,2 % des cas. Parmi les cas de dysplasie/métaplasie positifs pour le papillomavirus humain, 55,6 % étaient positifs pour les PVH 16 et 18, 22,3 % pour les PVH 6 et 11, et 11,1 % pour les PVH 31 et 33. Seuls les PVH 6 et 11 ont été dépistés dans quatre prélèvements biopsiques normaux (100 %).

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Introduction

Cervical cancer is one of the most prevalent forms of carcinoma among women worldwide and accounts for about 12% of all cancer cases among women with an incidence of more than 400 000 cases per year [*1*].

The association between certain human papillomaviruses (HPVs) and cervical cancer is well documented and research over the past 2 decades has convincingly revealed that HPVs are etiologically related to the development of most cases of cervical cancer [*2–4*]. Specific HPVs, most notably, HPV16 and HPV18 have been shown to be associated with cervical cancer.

In developing countries, screening for cervical cancer is based on the cytomorphologically abnormal epithelial cells in cervical smears. In spite of the success of cytomorphological examination in screening for cervical cancer, major limitations have been recognized, such as the low sensitivity [*5*]. Therefore, it has been proposed that HPV DNA testing be used to clarify equivocal pap smear screening results [*6,7*].

The presence of HPV in clinical specimens is usually established by molecular methods such as polymerase chain reaction (PCR), *in situ* hybridization and *in situ* PCR [*8*]. PCR is currently the most sensitive method for the detection of HPV DNA sequences in clinical specimens.

Cervical cancer like other forms of genital cancer is more prevalent in the northern part of the Islamic Republic of Iran including Mazandaran province [*9*]. An average of 450 cases of genital cancer are reported annually in this province [*9*]. In the year 2000, 456 cases of genital cancer were recorded by the Institute of Public Health Research, about 10% of which were cervical cancers [*9*]. However, the association between HPVs and cervical cancer has not been studied among Iranian women.

The main goal of our case–control study was to identify the prevalent types of HPV associated with cervical cancers in the Mazandaran province of the Islamic Republic of Iran using a PCR method.

Methods

Specimens

One hundred (100) samples were selected from archival, formalin fixed, paraffin embedded biopsy specimens of the cervix from Pathology Laboratory files in the north of the Islamic Republic of Iran. Forty-two (42) of the specimens were diagnosed cytomorphologically as cervical cancer, 14 of them were diagnosed as dys/metaplasia, and 44 of them were diagnosed as normal. The cervical cancer specimens included all grades of cervical neoplasia.

Specimens were sorted into various groups, including; normal, dys/metaplasia and cancerous by pathological results in parallel with haematoxylin and eosin staining of the biopsies.

DNA extraction

Sections of 5–10 μm wide were prepared from each specimen, avoiding any cross-contamination between samples (using separate disposable items such as gloves, blades and tubes; most importantly the first section of each specimen plus gloves and blade were discarded and new blade and gloves were used for main sectioning). Sections were subsequently deparaffinized by xylene and digested using digestion buffer containing proteinase K [*10*], followed by extensive extraction with phenol/chloroform [*11*]. The extracted DNA was stored at 4 °C until tested.

Extracted DNA from HeLa cell line was used as HPV-positive control. No DNA was added for negative controls.

PCR

DNA quality was evaluated by PCR using primers PCO3/PCO4 that amplify a 110 bp product from the human β-globin gene. β-globin positive samples were subjected to HPV PCR by GP5+/GP6+ primers for L1 open reading frame (ORF) that amplifies a 150 bp product from the HPV L1 ORF [*12*]. The World Health Organization generously provided all primers and they had following sequences:

- PCO3: 5′-ACACAACTGTGTTCACTAGC
- PCO4: 5′-CAACTTCATCCACGTTCACC
- GP5+: 5′-TTTGTTACTGTGGTAGATAC-TAC
- GP6+: 5′-AAAAATAAACTGTAAAT-CATATTC

PCR was performed according to the procedure described by Yi Ting et al. [*12*]. Samples were subsequently subjected to agarose gel electrophoresis (2% agarose), and stained with ethidium bromide.

In situ hybridization

PCR-based HPV-positive biopsy specimens were subjected to HPV typing using a commercially prepared *in situ* hybridization kit for detection of HPV types with separate probes obtained from DAKO (United States of America). For each specimen nine serial sections were processed, six of which were treated with biotinylated type-specific HPV probes (HPV6, 11, 16, 18, 31, 33). The three remaining sections were treated with a wide spectrum biotinylated HPV probe; a human DNA probe, and a plasmid probe in separate reactions and were used as controls.

Data processing

Data were processed by *Epi-Info 6* and all correlations were subjected to chi-squared test. Statistical significance was set as a *P*-value less than 0.05.

Results

Presence of HPV in cervical specimens, determined by PCR amplification

Cervical biopsy specimens were divided into three groups based on their pathology (Table 1). The results of this study show the presence of HPV in 78.6% of cervical carcinoma cases and 64.3% of dys/metaplasia cases, while HPV was only detected in 9% of normal cases. This observation clearly reconfirms earlier studies indicating a significant role for HPV in the pathogenesis of cervical carcinoma ($P < 0.05$). No significant correlation was observed between HPV presence and grade of cervical cancer ($P = 0.82$).

In situ hybridization genotyping of HPV

All 33 HPV-positive cancerous specimens reacted positively with the wide spectrum HPV probe. Specific probes for HPV6, 11, 16, 18, 31 and 33 reacted with 2 (6%), 4 (12.1%), 12 (36.4%), 8 (24.2%), 4 (12.2%), and 3 (9.1%) cervical cancer cases respectively (Table 2). The maximum level of reactivity was found with the HPV16 and HPV18 probes and the minimum level was found with the HPV6 probe. These results confirm the earlier studies that HPV16 and HPV18 are highly carcinogenic while HPV6 is regarded as a mild type.

Of the 9 HPV-positive dys/metaplasia specimens, 1 (11.1%) reacted with the HPV6-specific probe; this figure was 1 (11.1%) for HPV11, 3 (32.8%) for HPV16, 2 (22.2%) for HPV18, 1 (11.1%) for HPV31, and 1 (11.1%) was positive with the wide spectrum HPV probe but not reactive with any type-specific probes. Therefore, this isolate belonged to other

Table 1 **Human papillomavirus (HPV) status among study groups in Mazandaran province according to pathological findings**

Pathological findings	HPV-positive No.	%	HPV-negative No.	%	Total
Normal	4	9.1	40	90.9	44
Dys/metaplasia	9	64.3	5	35.7	14
Cancerous	33	78.6	9	21.4	42
Total	46	46.0	54	54.0	100

(undetermined) types of HPV. The maximum level of reactivity was found with the HPV16 and HPV18 probes and the minimum level with the HPV31 probe.

Of the 4 HPV-positive normal specimens, 3 (75%) were reactive with the HPV6-specific probe, and 1 (25%) was reactive with the HPV11-specific probe (Table 2). All HPV-positive dys/metaplasia and normal specimens (by PCR) were positive with the wide spectrum HPV probe. Reactivity with the HPV16 and HPV18 probes was not found in our normal specimens, while the maximum level of reactivity in cancerous specimens was found with these two types. These results further emphasize a significant correlation between the presence of highly carcinogenic HPV types (16 & 18) and the development of cervical carcinoma ($P = 0.001$).

Discussion

Cytological screening programmes using pap smears for the detection of cytomorphologically abnormal cells have dramatically decreased the number of deaths from cervical cancer, but have not eradicated the disease in any screened population to date [*5,13*]. Reports on failures of cytology to predict cervical cancer and cumulative

Table 2 **Human papillomavirus (HPV) genotyping of HPV-positive[a] samples of study group specimens in Mazandaran province according to pathological findings**

Pathological findings	HPV genotype														
	HPV6		HPV11		HPV16		HPV18		HPV31		HPV33		ND[b]		Total
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Normal	3	75.0	1	25.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	4
Dys/metaplasia	1	11.1	1	11.1	3	33.3	2	22.2	1	11.1	0	0.0	1	11.1	9
Cancerous	2	6.1	4	12.1	12	36.4	8	24.2	4	12.1	3	9.1	0	0.0	33
Total	6	13.0	6	13.0	15	32.6	10	21.7	5	10.9	3	6.5	1	2.2	46

[a]Determined by PCR.
ND = not determined.

false-negative error rates for invasive cancer of up to 50% have been published [*14,15*]. This complex cervical cancer detection system is prone to errors both in sampling (sampling error) and cytomorphological interpretation by cytologist (screening error) [*14*]. Additionally, with cytological screening, it remains unclear who is at increased risk for progression to cancer and how high- and low-risk groups should be evaluated, followed or treated. Accurate information about the presence or absence of HPV-related lesions is critical for future studies [*13*]. This poor predictive value of a negative cytological result suggests the need for better methods of detecting the presence of HPV-related lesions on the cervix.

Recent epidemiological and basic virological data strongly suggest that specific HPVs play an important role in the development of cervical cancer [*16*]. In general, HPV6 and 11 have been associated with benign cervical lesions and are referred to as non-oncogenic or low-risk HPV types, whereas HPV16 and 18 and to a lesser extent HPV31, 33 and 35 have been associated with cervical lesions with severe dysplasia, high-grade CIN, and have been found in about 90% of cervical carcinomas [*5,17,18,19*]. These latter HPVs are regarded as oncogenic or high-risk HPV types.

As HPV cannot be cultured *in vitro*, analysis of DNA sequences can be used to identify HPV genotypes. PCR and *in situ* hybridization are two of the most sensitive methods [*20,21*]. The detection of HPV in cervical biopsies using these methods in conjunction with cytology could potentially improve screening for cervical cancer. In this study we used the PCR method for initial detection of HPV and *in situ* hybridization was subsequently used for genotyping of HPVs.

Of the 100 specimens examined in this study, 42 were cervical carcinoma cases, 14 dys/metaplasia cases and 44 normal cases. Based on the initial PCR screening, 33 (78.6%) cases were HPV-positive among the cancerous group, 9 (64.3%) cases were HPV-positive among the dys/metaplasia group, while 4 (9%) cases were positive among the normal group (Table 1). Although a significant correlation was observed between HPV infection and cervical cancer ($P < 0.05$) no significant correlation was observed between presence of HPV and grade of cervical cancer ($P = 0.44$).

The detection of HPV DNA sequences in 78.6% of cervical cancer biopsy specimens in our study is similar to other studies which have reported detection rates ranging from 66% to 94% [*22–27*].

In our study, genotyping of HPV isolates by *in situ* hybridization revealed that HPV types 16 and 18 were the most commonly encountered types among cervical carcinoma HPV-positive cases and among dys/metaplasia HPV-positive cases, while they were not found in the normal group (Table 2). Based on our results, HPV types 16 and 18 accounted for 60.6% and 55.6% of HPV infections among the cervical carcinomas and dys/metaplasia examined respectively, while these types were never detected in normal cases. HPV types 6 and 11 accounted for 18.2% and 22.21% of HPV infections among the cervical carcinomas and dys/metaplasia, respectively. HPV types 31 and 33 were detected in 21.2% of HPV-positive cervical carcinoma cases and only one HPV31 case was detected in HPV-positive dys/metaplasia cases, while only HPV types 6 and 11 were detected in all of the normal HPV-positive cases. We also encountered a case of undetermined type of HPV among the dys/metaplasia cases, which was absent in the

normal and cancerous cases. Detection of undetermined types of HPV in cervical infections is unusual and their significance needs to be further examined. Similar results were reported in an Australian study in which HPV types 16 and 18 were detected in 53.8% and 17.2% of HPV-positive cases respectively. In that study, 21% of HPV-positive cases were attributed to other HPV types [*23*]. In South African women, HPV18 was detected in 34% of SCC and 19.4% of CIN cases [*22*].

Our results strongly confirm a significant correlation between specific types of HPV and the development of cancer in a region of the Islamic Republic of Iran with a high prevalence of cancer in general ($P < 0.05$). These results revealed that cervical tumours in the Mazandaran province are most commonly caused by HPV types 16 and 18 and to a lesser extent by HPV31 and HPV33. It is estimated that invasive cervical cancer takes about 12 years to develop [*5*]. Since cervical specimens may appear to be normal upon initial cytopathological screening, detection of HPV types 16 and 18 DNA sequences may be used to predict likely development of cervical cancer in the future. The presence of HPV types 6 and 11 probably would not predict a risk for developing cervical tumours.

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