Article is available online at http://www.webio.hu/por/2003/9/2/0121

ARTICLE

High Risk HPV Types in Southern Iranian Patients with Cervical Cancer

S FARJADIAN,[1] E ASADI,[2] M DOROUDCHI,[1,3] A SAMSAMI DEHAGHANI,[4] SZ TABEI,[2] VP KUMAR[2] A GHADERI[1,3]

[1]Department of Immunology, [2]Department of Pathology, [3]Shiraz Institute for Cancer Research, [4]Department of Obstetrics and Gynecology, Shiraz University of Medical Sciences, Shiraz, Iran

This study was undertaken to assess the rate of HPV infection in cervical carcinoma among southern Iranian patients. 101 archival cervical carcinoma tissue samples of a 10 year period were studied for the presence of HPV DNA in southern Iran by a polymerase chain reaction method. In addition, the presence of high risk HPV-16 and HPV-18 genotypes was investigated. In total, 88 (87.1%) of the samples were HPV DNA positive, of which 83 were squamous cell carcinomas and 5 were adenocarcinomas. HPV-16 genotype was detected in 26.7% of HPV positive cervical carcinomas; however, none of the samples were positive for the existence of HPV-18 genotype. Collectively, these results suggest that HPV-16 and HPV-18 are not the frequent high risk HPV types in our patients and circulating HPV types in southern Iranian population are different from many other populations. (Pathology Oncology Research Vol 9, No 2, 121–125, 2003)

Keywords: cervical cancer, HPV

Introduction

Cervical cancer is the second most prevalent cancer among women worldwide.[1] However, the prevalence of cervical cancer in Iran is much lower than in the other parts of the world. In 1998, cervical cancer with the prevalence rate of 0.71 per 100000 of population ranked the 14th common cancer in Fars province, Iran.[2] There is a huge number of publications addressing human papilloma virus (HPV) as the major risk factor of developing cervical cancer.[3,4] The E6 and E7 proteins of the virus are known to interfere with cell cycle regulators.[5] In addition, integration of viral genome into the host DNA results in E6 and E7 protein overexpression which inactivate p53 and retinoblastoma proteins of host cells.[5,6] These events collectively result in immortalization of cells and uncontrolled cell proliferation.

Several high risk strains of HPV are known to be more associated with cervical cancer among which, HPV-16 and HPV-18 are the most common reported genotypes. According to the International Agency for Research on Cancer (IARC), these two HPV types are classified as carcinogens in humans.[7] It is also reported that HPV-16 integration is significantly associated with invasiveness of genital tumors.[8] The other known risk factors of cervical cancer are high parity, smoking, sexual behavior and hygiene, which are known to modify the risk in women infected with HPV.[9-11]

It is estimated that 80% of new cases occur in developing countries.[1] The incidence of cervical cancer in developed countries is much less than in developing countries which seems to be related to the screening programs in the developed world. It is suggested that biennial screening for HPV combined with Pap tests can have life saving benefits compared to Pap smears alone.[12] Despite the observed close association between HPV-16/18 genotypes and cervical cancer, these two HPV types are not always the most common genotypes among HPV-infected women and/or patients with cervical cancer.[13] Previous studies have postulated that HPV genotypes may vary according to the geographic region.[13] Therefore, having information on the circulating genotypes of HPV and knowing the high risk HPV types in each population will help to perform such screening tests more efficiently.

Received: April 29, 2003; *accepted:* June 15, 2003

Correspondence: Abbas GHADERI, Shiraz Institute for Cancer Research (ICR), Shiraz University of Medical Sciences, P.O. Box: 71345-3119, Shiraz, Iran. Tel/fax: +98-711-230 3687
email: ghaderia@sums.ac.ir

To our best knowledge there is no official report on the rate of HPV infection among Iranian patients with cervical carcinoma. Therefore, this study was undertaken to assess the rate of HPV infection and its high risk genotypes in Southern Iranian patients with cervical cancer.

Materials and Methods

Tissue samples

A total of 101 formalin-fixed paraffin-embedded blocks of patients with histologically proven cervical cancer obtained from archival specimens of Pathology Department, Shiraz University of Medical Sciences during a 10 years period (1991-2000) were included in this study. Mean age of patients was found to be 49.1 years. *Table 1* illustrates the characteristics of tumors. There was no complete information available about parity and smoking status of patients.

Table 1. **Characteristics of tumors**

	Number	*Type16 (%)*	*HPV negative (%)*	*Other types (%)*
Histological type				
SCC	93	22 (23.7)	10 (10.7)	61 (65.6)
AC	8	1 (12.5)	3 (37.5)	4 (50)
FIGO stage				
I	6	2 (33.3)	2 (33.3)	2 (33.3)
II	10	1 (10)	2 (20)	7 (70)
III	5	0	1 (20)	4 (80)
IV	0	0	0	0
Not determined	80	20 (25)	8 (10)	52 (65)
Differentiation				
CIN	16	2 (12.5)	2 (12.5)	12 (75)
Well	22	3 (13.6)	2 (9.1)	17 (77.3)
Moderate	16	3 (18.7)	4 (25)	9 (56.3)
Poor	6	2 (33.3)	2 (33.3)	2 (33.3)
Not determined	41	13 (31.7)	3 (7.3)	25 (61)

DNA preparation

DNA was extracted from paraffin-embedded cervical tissues as follows: 10 µm thick dry sections were sliced from each samples and placed in sterile 2 ml Eppendorf tubes. To avoid carry-over of samples the microtome blade was carefully cleaned with xylene between sectioning of blocks. Paraffin was removed with two rounds of warm xylene extraction (60°C) followed by two 90% ethanol washes. After high-speed centrifugation of the tissues, samples were incubated in digestion buffer (10 mM Tris-Cl, pH=8.5, 25 mM EDTA, 0.5% SDS and 2 mg/ml Proteinase K) for 5 days at room temperature with shaking. An equal volume of phenol: chloroform (1:1) was then added, mixed gently and centrifuged. The aqueous phase was mixed with an equal volume of chloroform. DNA was extracted from aqueous phase by adding an equal volume of isopropanol in the presence of 30 µl of 3 M Na-acetate (pH=5.2). The precipitated DNA was washed twice with 70% ethanol to remove salt. The pellets were vacuum-dried for 15 min and redissolved in deionized distilled water.

HPV DNA detection

The primer set described by Snijders et al.[14] 5'TTTGTTACTGTGGTAGATAC3' and 5'GAAAAATAAACTGTAAATCA3' which amplified a 140 bp fragment was used for amplification of a common region in all HPV types in a touch down polymerase chain reaction (PCR). Approximately 0.25 µg of extracted DNA was amplified in each 25 µl PCR reaction containing 200 mM of each dNTPs (Boehringer, Germany), 1 U of Taq DNA polymerase (Sinagen, Iran), 2.5 µl of 10X PCR buffer, 20 pmol of each primer (TIB MOLBIOL, Germany). The reaction mixture was first heated at 94°C for 4 min and amplification was done for 40 cycles using a touch down PCR program. For the first 7 steps annealing temperature was reduced 2 degrees every 2 cycles from 54°C to 42°C. Further 26 cycles were done at annealing temperature equal to 40°C for 1 min. The denaturation and extension in all cycles were done for 1 min each at 94°C and 72°C, respectively. The products were then overextended for further 5 min at 72°C and kept at 4°C until further use. The amplified fragments were resolved by electrophoresis on the 2% agarose gel in 80 volts for 90 min and stained with ethidium bromide and visualized on a UV. transilluminator (Uvitec, UK).

DNA samples positive for HPV DNA were selected for a further amplification with two pairs of type-specific primers to unique sequences of the E6 regions of HPV-16 and HPV-18 as described by Samoylova et al.[15] The primer sequences were as follows:

HPV-16/F 5' TCA AAA GCC ACT GTG TCC TG 3'
HPV-16/R 5' CGT GTT CTT GAT GAT CTG CA 3'
HPV-18/F 5' GAC ACA TTG GAA AAA CTA AC 3'
HPV-18/R 5'TAG TGC CCA GCT ATG TTG TG 3'

PCR reaction was performed in a total volume of 25 µl containing 2.5 µl of 10X PCR buffer, 2 mM $MgCl_2$, 200 mM of each dNTPs, 1U of Taq DNA polymerase, 20

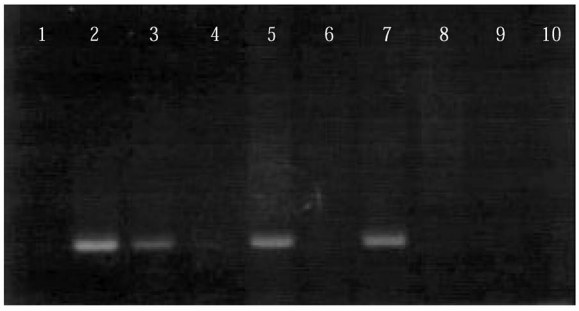

Figure 1. *PCR analysis of DNA samples extracted from cervical biopsies using HPV common primers: lane 1: DNA extracted from peripheral blood of a healthy individual; lane 2: pHPV-18; lanes 3, 5, and 7: cervical biopsies containing HPV genome; lanes 4, 6, 8, 9 and 10: cervical biopsies with no HPV genome.*

pmol of each primers (a mixture of HPV-16 and HPV-18 specific primers). 20 ng of HPV DNA as positive control, or 500 ng of extracted DNA from a healthy individual as negative control, or 500 ng of extracted DNA from each samples was added to the reaction mixture. After holding at 94°C for 3 min the mixture was subjected to 30 cycles of PCR amplification (denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min). The last cycle was followed by an additional 5 min of extension at 72°C. The amplified fragments were resolved by electrophoresis on the 2% agarose gel in 80 volts for 90 min and stained with ethidium bromide and visualized on a UV. transilluminator (Uvitec, UK).

Results

Tissue specimens

In this study 101 tissue samples with confirmed histopathological characteristics of cervical cancer were studied. According to the FIGO classification, 93 (92.1%) out of 101 samples were squamous cell carcinomas and 8 (7.9%) out of 101 were diagnosed as adenocarcinomas. Of the 93 squamous cell carcinomas, 16 (17.2%) were carcinoma in situ (CIN) and 77 (82.8%) were invasive carcinomas.

HPV prevalence and genotyping

In total, 88 (85.1%) out of 101 samples were revealed to be positive for the existence of the specific band amplified by common HPV primers in the PCR (***Figure 1***). HPV genome was detected in 83 (89.3%) out of 93 squamous cell carcinoma cases and 5 (62.5%) out of 8 adenocarcinoma cases. Among the 86 HPV positive samples 23 (26.7%) were positive for HPV-16 DNA while none of them were positive for HPV-18 DNA. ***Figure 2*** illustrates the results of PCR analysis for HPV-16 and HPV-18 genotypes. Of the 23 HPV-16 positive samples, 22 (95.7%) were squamous cell carcinomas and 1 (4.3%) was adenocarcinoma.

Discussion

Human papillomavirus is a DNA virus, and its association with cervical cancer is strongly suggested. Certain types of the virus are considered as high risk types due to the great odds ratios of association with cervical cancer and their ability to integrate into the host genome.[16] In the present study we investigated the rate of HPV infection among southern Iranian cervical cancer patients and the frequency of two HPV high risk types in these patients. In total, 87.1% of studied samples were positive for the presence of HPV DNA, which was comparable with the results of some previous studies in Danish, Belgian, Brazilian and Chinese patients.[17-20] Genotyping for HPV-16 and HPV-18 revealed that 26.7% of HPV positive samples were HPV-16 DNA positive, however, none of them were positive for HPV-18 DNA. The very low rate of HPV-16 infection in cervical cancer is contrary to the previous reports of high infection rate of this high risk type in different populations (***Table 2***).[17,19,21] However, it is comparable to the results of some other studies.[13,18,22] The study of Castellsague in Mozambique[13] has shown that there may be differences in the dominant HPV types in different populations and regions. In this regard, the report of higher HPV-16 titers in carriers of a specific HLA-DRB1-DQB1 haplotype is noteworthy.[23] This is especially interesting because of the importance of HLA class II molecules in the evoking of immune response to the HPV virus and the importance of

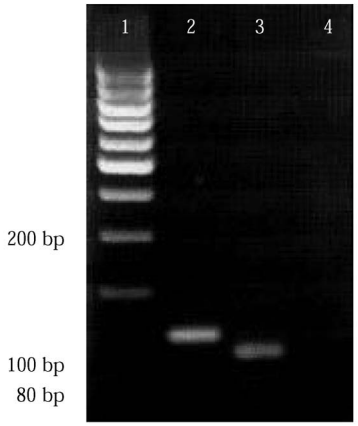

Figure 2. *PCR analysis of DNA samples extracted from cervical biopsies using two sets of type-specific primers for HPV-16 and HPV-18: lane 1: DNA size marker; lane 2: pHPV-18; lane 3: extracted DNA from cervical biopsy containing HPV-16; lane 4: extracted DNA from peripheral blood of a healthy individual.*

Table 2. **Comparison of the frequency of HPV infection in cervical carcinoma of different populations**

Population or country	*Total HPV (%)*	*HPV-16 (%)*	*HPV-18 (%)*	*Reference (no.)*
Alaska natives	98.1%	78.8%	3.8%	Sebbelo, et al. (17)
Eskimos	96.8%	80.8%	3.3%	Sebbelov et al. (17)
Aleut & Indian	100%	77.3%	4.5%	Sebbelov et al. (17)
Greenland natives	84.4%	96.3%	0	Sebbelov et al. (17)
Danish Caucasians	85.3%	82.8%	0	Sebbelov et al. (17)
Australia	91.9%	53.8%	17.2%	Chen et al. (21)
Senegal	94%	42%	39%	Chabaud et al. (22)
Hong Kong	83.7%	79.6%	7.5%	Lo et al.(19)
Chinese	83.7%	61.7%	14.8%	Lo et al. (19)
Southern Iranian	87.1%	26.7%	0	Current study

HLA class II binding peptides in the design of peptide-based vaccines.[24] In a previous report on the frequency of HLA class II alleles in a limited number of cervical cancer patients, we observed an association between HLA DQB1*0601 and Squamous Cell Carcinoma of cervix.[25] In that study only one of our patients and none of the controls had DRB1*1501-DQB1*0601/0602 haplotype. In addition, the frequencies of HLA-DRB1*1501 and HLA-DQB1*0602 were 6.0% and 8.5%, respectively in southern Iranian normal population. In addition, the frequency of DRB1*1501-DQB1*0602 haplotype is 2.5% in southern Iranian normal individuals.[26] The low rate of HPV-16 infection in our cervical cancer patients is consistent with the low frequencies of HLA-DRB1*1501 and HLA-DQB1*0602 alleles in our population.

Of the total 101 tested and 86 HPV positive samples, none were positive for HPV-18 DNA. This is contrary to the high rate of HPV-18 detection in Australia, Senegal and Hong-Kong.[18,20,21] HPV-18 is mostly found in adenocarcinoma of cervix and has a low rate in squamous cell carcinoma of cervix.[27,28] Since only 8 (7.9%) of 101 samples in this study were adenocarcinomas, it is logical to assume that not having HPV-18 genotype is due to the low adenocarcinoma case numbers. However, it can also suggest that other HPV high risk genotypes are the dominant types in our patients.

In conclusion, our study, which is the first report on the HPV types in Iranian cervical cancer patients, suggests that HPV high risk types among southern Iranian population are different from most other countries. Currently the circulating HPV types in our population are under investigation.

Acknowledgement

This work was financially supported by Shiraz Institute for Cancer Research and a grant (grant number 77-571) from Shiraz University of Medical Sciences, Shiraz, Iran.

References

1. *Parkin DM:* Global cancer statistics in the year 2000. Lancet Oncol 2:533-543, 2001
2. *Farahmandbeigi M, Kadivar MR:* The incidence rate of registered cancer in Fars province. Disease Control Unit, Shiraz University Press, Iran, 2000
3. *Bosch FX, Munoz N.* The viral etiology of cervical cancer. Virus Res 89:183-190, 2002
4. *Walboomers JM, Jacobs MV, Manos MM, et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189:12-19, 1999
5. *Southern SA, Herrington CS.* Disruption of cell cycle control by human papillomaviruses with special reference to cervical carcinoma. Int J Gynecol Cancer 10:263-274, 2000
6. *Park TW, Fujiwara H, Wright TC.* Molecular biology of cervical cancer and its precursors. Cancer 76:1902-1913, 1995
7. IARC working Group, Human papilloma viruses, IARC Monograph on the evaluation of carcinogenic risks to humans. Lyon, France: International Agency for Research on Cancer, 65, 1995
8. *Badaracco G, Venuti A, Sedati A, Marcante ML.* HPV16 and HPV18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. J Med Virol 67:574-582, 2002
9. *Munoz N, Franceschi S, Bosetti C, et al.* International Agency for Research on Cancer. Multicentric Cervical Cancer Study Group Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. Lancet 359:1093-1101, 2002
10. *Castle PE, Wacholder S, Lorincz AT, et al.* A prospective study of high-grade cervical neoplasia risk among human papillomavirus-infected women. J Natl Cancer Inst 94:1406-1414, 2002
11. *Rong S, Chen W, Wu L, et al.* Analysis of risk factors for cervical cancer in Xiangyuan County, Shanxi Province Zhonghua Yu Fang Yi Xue Za Zhi 36:41-43, 2002
12. *Mandelblatt JS, Lawrence WF, Womack SM, et al.* Benefits and costs of using HPV testing to screen for cervical cancer. JAMA 287:2372-2381, 2002
13. *Castellsague X, Menendez C, Loscertales MP, et al.* Human papillomavirus genotypes in rural Mozambique. Lancet 358:1429-1430, 2001

14. *Snijders PJ, van den Brule AJ, Schrijnemakers HF, et al.* The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol 71:173-181, 1990
15. *Samoylova EV, Shaikhaiev GO, Petrov SV, et al.* HPV infection in cervical cancer cases in Russia. Int J Cancer 61:337-341, 1995
16. *Ferenczy A, Franco E.* Cervical-cancer screening beyond the year 2000. Lancet Oncol 2:27-32, 2002
17. *Sebbelov AM, Davidson M, Kruger Kjaer S, et al.* Comparison of human papillomavirus genotypes in archival cervical cancer specimens from Alaska natives, Greenland natives and Danish Caucasians. Microbes Infect 2:121-126, 2000
18. *Goncalves MA, Massad E, Burattini MN, Villa LL.* Relationship between human papillomavirus (HPV) genotyping and genital neoplasia in HIV-positive patients of Santos City, Sao Paulo, Brazil. Int J STD AIDS 10:803-807, 1999
19. *Lo KW, Wong YF, Chan MK, et al.* Prevalence of human papillomavirus in cervical cancer: a multicenter study in China. Int J Cancer 100:327-331, 2002
20. *Baay MF, Tjalma WA, Weyler J, et al.* Human papillomavirus infection in the female population of Antwerp, Belgium: prevalence in healthy women, women with premalignant lesions and cervical cancer. Eur J Gynaecol Oncol 22:204-208, 2001
21. *Chen S, O'Sullivan H, Tabrizi SN, et al.* Prevalence and genotyping of HPV in cervical cancer among Australian women. Int J Gynaecol Obstet 67:163-168, 1999
22. *Chabaud M, Le Cann P, Mayelo V, et al.* Detection by PCR of human papillomavirus genotypes in cervical lesions of Senegalese women. J Med Virol 49:259-263, 1996
23. *Beskow AH, Gyllensten UB.* Host genetic control of HPV 16 titer in carcinoma in situ of the cervix uteri. Int J Cancer 101:526-531, 2002
24. *Zwaveling S, Ferreira Mota SC, Nouta J, et al.* Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol 169:350-358, 2002
25. *Dehaghani AS, Amirzargar A, Farjadian S, Ghaderi A.* HLA-DQB1 alleles and susceptibility to cervical squamous cell carcinoma in Southern Iranian patients. Pathol Oncol Res 8:58-61, 2002
26. *Amirzargar A, Mytilineos J, Farjadian Sh, et al.* Human Leukocyte Antigen class II allele frequencies and haplotype association in Iranian population. Hum Immunol 62:1234-1238, 2001
27. *Hording U, Teglbjaerg CS, Visfeldt J, Bock JE.* Human papillomavirus types 16 and 18 in adenocarcinoma of the uterine cervix. Gynecol Oncol 46:313-316, 1992
28. *Bjersing L, Rogo K, Evander M, et al.* HPV 18 and cervical adenocarcinomas. Anticancer Res 11:123-127, 1991