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RESEARCH

Human Papilloma Virus Early Proteins E6 (HPV16/18-E6) and the Cell Cycle Marker P16 (INK4a) are Useful Prognostic Markers in Uterine Cervical Carcinomas in Qassim Region- Saudi Arabia

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Abstract Cervical cancer is a common and an important public health problem for adult women in developing countries. In contrast, cervical cancer incidence is low in Saudi Arabia. High-risk types of human papilloma viruses (HPV16 and HPV18) are the most significant risk factors for cervical cancer. HPV16/18-E6 oncoprotein is associated with HPV etiology, viral persistence and epithelial transformation. Cell cycle protein p16 INK4a (p16) plays an important role in the pathophysiology of cervical carcinomas. The aims of this study were to investigate the expression of HPV16/18-E6 and p16 in uterine cervical carcinomas in Qassim Region - Saudi Arabia, and to relate the results to the established clinicopathological prognostic parameters (age of the patient, educational level, birth control methods, number of pregnancy, smoking status, degree of histological differentiation, clinical stage, and lymph node metastasis) The study included 40 specimens of uterine cervical squamous cell carcinomas diagnosed and confirmed by biopsy. Histopathological classification of cervical tumors cases was performed according to the International Federation of Gynecology and Obstetrics (FIGO). Immunohistochemical analysis for HPV16/18-E6 and p16 were carried out on formalin-fixed paraffin-embedded sections of cervical tissues using avidin-biotin peroxidase method. There was a significant statistical correlation between HPV16/18-E6 expression in cervical carcinoma and nationality, smoking status and size of the tumor. HPV16/18-E6 oncoprotein expression in normal lymphocytes and endothelial cells in the tumor tissues and the adjacent normal cervical tissues suggest the possibility that HPV infection might spread to other organs through blood circulation. P16 expression has been correlated with high grade, stage of cervical SCC and HPV16/18-E6 expression. The current study supports the critical function of p16 and HPV16/18-E6 as specific markers for cervical carcinoma. However the potential for usage of p16 and HPV16/18-E6 as prognostic markers will require detailed follow data for a larger group of patients.

Keywords Immunohistochemistry · Cervical carcinoma · HPV16/18E6 · P16 (INK4a)

Introduction

Worldwide, cervical cancer is an important public health problem for adult women [1, 2]. The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) estimated that nearly 80 % of cervical cancer cases occur in developing countries and, in many such regions, it is the most common cancer and cause of death from cancer among women [3]. The vast majority of cervical cancer cases are caused by infection with certain subtypes of human papilloma virus (HPV), a sexually transmitted virus that infects cells and may result in precancerous lesions and invasive cancer [4, 5]. The worldwide prevalence of HPV is 10.4 % [6]. Women are generally infected with HPV in their teens; it can take as long as 20 years after HPV infection for the cancer to develop. The prevalence of cervical HPV infection decreases sharply in women after the age of 30 [7]. The American

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Cancer Society provides the following list of risk factors for cervical cancer: Human Papillomavirus (HPV) infection, smoking, HIV infection, chlamydia infection, dietary factors, oral contraception, multiple pregnancies, exposure to the hormonal drug diethylstilbestrol (DES) and a family history of cervical cancer. These factors probably modify the risk in women infected with HPV [8, 9]. Malignant transformation of the cervical epithelial cells is associated with persistent high-risk HPV infections, such as HPV 16 and 18 which encode E6 and E7 oncoproteins that bind to and inactivate the tumor suppressor proteins p53 and pRB (retinoblastoma protein), respectively [10].

In contrast, cervical cancer incidence is very low in Saudi Arabia, ranking number 11 between all cancers in females [11]. The closed society, standards of mores and male circumcision might reduce women exposure to HPV infection [12–16]. The prevalence of HPV infection among women and its association with cervical cancer in Saudi Arabia and in similar socio-cultural societies is scanty [17–21]. Discordant reports showed high incidence in some countries, such as Indonesia [22, 23]. Inherited genetic predisposition may contribute to the risk of cervical cancer. Tumor suppressor gene TP53 has been suggested to affect the oncogenic potential of the HPV E6 protein [24]. Recent study reported that activation of interleukin-6/signal transducer and activator of transcription 3 by HPV16/18-E6 induces fibroblast senescence to promote cervical tumourigenesis through autocrine and paracrine pathways in tumor microenvironment [25]. Moreover, HPV16 oncoproteins promote invasiveness of cervical cancer by upregulating specific matrix metalloproteinases [26].

P16 is part of cascade cell cycle regulators, which control the transition cell from G1 to S phase [27]. Cyclin D and cyclin-dependent kinases (CDK) make complexes that phosphorylate pRB and ensure cell proliferation to which p16 has a negative regulation role [28]. Many studies indicated the potential of this biomarker for cervical carcinoma diagnosis and prognosis.

An association between HPV16/18-E6 and p16 expression in human cervical squamous cell carcinoma in Qassim Region-Saudi Arabia has not been studied. Therefore, we analyzed immunohistochemical expression of HPV16/18E6 and P16 in cervical carcinoma to examine their relationship and to determine any association with other existing clinico-pathological parameters.

Patients and Methods

This study was carried out with cooperation of Pathology Department, King Fahd Specialist Hospital, Buraidah, and other hospitals in Qassim region. The retrospective study was conducted on cervix uteri specimens obtained from cervical carcinoma patients from December 2000 to March 2013. Patients' clinical information including age, nationality, smoking status, number of pregnancies, the main complaints, the clinical presentations was obtained from electronic medical records.

Histopathology

Specimens were examined, diagnosed, and classified according to the International Federation of Gynecology and Obstetrics (FIGO) of cervical tumors. The normal cervical epithelium was obtained from areas adjacent to the tumor tissues. The tumor size, site and number were recorded and analyzed. The tissue specimens were fixed in 10 % formalin, dehydrated and embedded in paraffin and stained with hematoxylin and eosin (H&E). The slides were then examined for histological diagnosis and assessment.

Immunohistochemistry

From the formalin fixed paraffin-embedded tissues, 4 μ sections were immunostained with monoclonal antibodies against HPV16E6/18-E6 (C1P5) (Santa Cruz Biotechnology, Inc. USA, dilution 1:50) and p16 (F-12) (Santa Cruz Biotechnology, Inc. USA, dilution 1:50) using labeled streptavidin biotin (LSAB) method. Sections were cut and dried at 37 °C, dewaxed in xylene and rehydrated using serial concentrations of ethanol and washed in running water. Sections were washed with Tris buffer and preincubated with normal rabbit serum (10 %) for 20 min. Slides were incubated with the monoclonal primary antibodies for one hour and exposed to ImmunoCruz™ mouse LSAB Staining (Santa Cruz Biotechnology, Inc. USA). The slides were then counterstained with hematoxylin and reviewed. Known positive control sections were included in each run to ensure proper immunostaining whereas the negative control consisted of the same section where the primary antibody was omitted. Sections of the p16INK4a-positive human uterine adenocarcinoma tissue and HPV16/18 E6-positive HPV-positive vulva carcinoma were included to serve as the positive controls for p16INK4a and HPV16/18 E6 IHC, respectively. Dichotomic negative/positive evaluation was adapted to determine E6 immunoreaction as suggested by Lin et al. with some modification [29]. Brown nuclear or cytoplasmic staining was considered as a positive reaction to E6 HPV 16/18 proteins. Immunohistochemical expression for p16INK4a was quantified according to staining intensity (weak, moderate and strong) and dispersion pattern (diffuse or focal) in each block [30].

Statistical Analysis

Statistical calculations were performed by using Statview programme package. Data were compared using Chi-square test, and the differences between the means of continuous data

will be compared by using paired *t*-test. The percentage of expression of HPV16 E6/18-E6 and p16 in malignant cervical squamous cell carcinoma, were used to assess the role of (FIGO)'s classification, differentiation, age, and tumor size. A probability p value of <0.05 was considered statistically significant.

Results

Clinical and Histopathological Data

This study included 40 cases of cervical carcinoma. The study included 21 Saudi women and 19 non-Saudi women. The patient ages ranged from 28 years to 76 years, (median for Saudi women=50 and for non-Saudi women=35). The non Saudi women showed development of carcinoma at younger ages compared to Saudi women (p <0.05). The clinical presentations were mostly vaginal bleeding for more than one month. Forty eight percent of the patient were smokers and 35 % either take or having a history of contraceptive pills administration. Histologically, the specimen were classified as 14 cases (35 %) well differentiated, 19 cases (47.5 %) moderately differentiated and 7 cases (17.5 %) poorly differentiated cervical squamous cell carcinoma.

HPV16/18-E6 Oncoprotein is Expressed in Cervical Squamous Cell Carcinoma

To explore whether HPV16 infection could be linked to cervical carcinoma development in Qassim region, Saudi Arabia, HPV16/18-E6 oncoprotein expression was evaluated by immunohistochemistry in cervical SCC. Our data showed HPV 16/18-E6 positivity in 21cases (52.5 %); 73.6 % of positive cases were non Saudi (p <0.05). The staining reaction was seen cytoplasmic and nuclear. Meanwhile, most of the mature squamous epithelial cells were negative. Most malignant squamous cells showed diffuse expression (Fig. 1). The accompanied squamous dysplasia in the studied sections also showed positive immunoreactivity for the HPV16/18-E6; it was seen positive mainly in the superficial epithelial cells. There was statistically significant correlation between HPV16/18-E6 expression and smoking status, where more positive expression were associated with smoking (p <0.011). There was statistically significant correlation between HPV16/18-E6 expression and tumor size, where it was more commonly expressed in tumor diameter more than 2 cm. There was no statistically significant correlation between HPV16/18-E6 expression and age of the patient, educational level, contraceptives administration, number of pregnancies, grade and stage of the tumor and lymph node metastasis (p >0.05) (Tables 1 and 2).

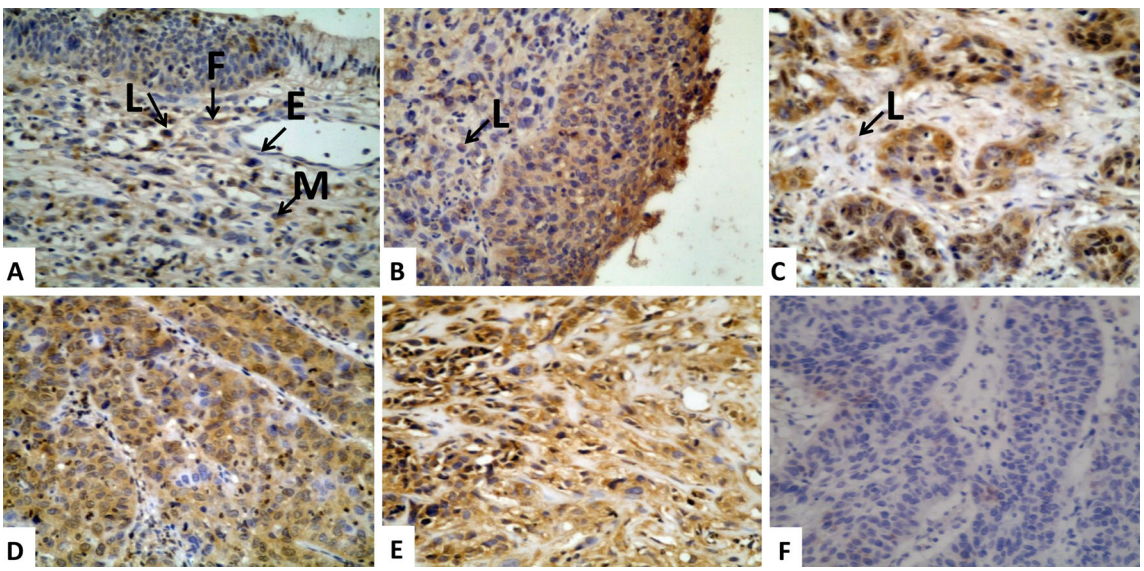

Fig. 1 Immunohistochemical analysis of human papilloma virus 16/18-E6 protein in cervical SCC, and adjacent normal tissues. **A** a negative HPV16/18-E6 immunostaining in normal cervical epithelial cells, and positive expression in lymphocytes, macrophages, fibroblast and endothelial cells (×200); **B** HPV16/18-E6 protein expressed in lymphocytes and in carcinoma in situ tumor cells (×200); **C**: HPV16/18-E6 expressed in well differentiated SCC and lymphocytes in the stroma (×400); **D** HPV16/18-E6 expressed in moderately differentiated SCC (×400); **E** HPV16/18-E6 expressed in poorly differentiated SCC (×400); **F** a negative HPV16/18-E6 immunostaining in tumor cells of moderately differentiated SCC sheets (×400) (L=lymphocyte, F=fibroblast, E=endothelial cell, M=macrophage)

Table 1 The correlation between HPV16/18-E6 and p16 protein expression in cervical squamous cell carcinoma and the clinical parameters

		HPV16/18 E6 Expression			p16 Expression							
						Intensity				Dispersion		
Samples	Total	− (%)	+(%)	p	− (%)	weak (%)	moderate (%)	Strong (%)	p	Focal (%)	Diffuse (%)	p
SCC	40	19 (47.5)	21 (52.5)		8 (20)	10 (25)	17 (42.5)	5 (12.5)		9 (22.5)	23 (57.5)	
1. Age (Years)												
<45	22 (55)	10 (45.5)	12 (54.5)	0.781	5 (22.7)	5 (22.7)	8 (36.4)	4 (18.2)	0.858	6 (27.3)	11 (50)	0.224
>45	18 (45)	9 (50)	9 (50)		3 (16.7)	5 (27.7)	9 (50)	1 (56)		3 (16.3)	12 (66.7)	
2. Nationality												
Saudi	21 (52.5)	14 (66.4)	7 (33.3)	0.024 (S)	4 (9)	7 (33.3)	9 (42.9)	1 (4.8)	0.29	4 (19.1)	13 (61.9)	0.411
None-Saudi	19 (47.9)	5 (26.3)	14 (73.7)		4 (21.1)	3 (15.8)	8 (42.1)	4 (21.1)		5 (26.4)	10 (52.6)	
3. Educational level												
No formal Education	18 (45)	10 (55.6)	8 (44.4)	0.796	3 (16.7)	5 (27.8)	8 (44.4)	2 (11.1)	0.454	5 (27.7)	10 (55.6)	0.893
Basic Education	16 (40)	6 (37.5)	10 (62.5)		2 (12.5)	4 (25)	8 (50)	2 (12.5)		4 (25)	10 (62.5)	
Higher education	6 (15)	3 (50)	3 (50)		3 (50)	1 (16.7)	1 (16.7)	1 (16.7)		0 (0)	3 (50)	
4. Smoking status												
No	21 (52.5)	14 (66.5)	7 (33.5)	0.011 (S)	3 (14.3)	6 (28.6)	11 (52.4)	1 (4.7)	0.978	5 (23.8)	13 (61.9)	0.677
Yes	19 (47.5)	5 (26.3)	14 (73.7)		5 (26.2)	4 (21.1)	6 (31.5)	4 (21.1)		4 (21.1)	10 (52.6)	
5. Contraceptives												
No	26 (65)	15 (57.9)	11 (42.3)	0.132	6 (23.1)	6 (23.1)	12 (46.2)	2 (7.6)	0.424	6 (23.1)	14 (53.8)	0.479
Yes	14 (35)	4 (28.6)	10 (71.4)		2 (14.3)	4 (28.6)	5 (35.7)	3 (21.4)		3 (21.4)	9 (64.3)	
6. Number of pregnancies												
No	9 (22.5)	4 (44.4)	5 (55.6)	0.659	0 (0)	1 (11.1)	5 (%)	3 (%)	0.235	3 (33.3)	6 (66.7)	0.648
1–2	11 (27.5)	5 (45.5)	6 (54.5)		4 (36.4)	3 (27.2)	4 (36.4)	0 (0)		3 (27.2)	4 (36.4)	
3–4	10 (25)	6 (60)	4 (40)		3 (30)	2 (20)	4 (40)	1 (10)		1 (10)	6 (60)	
>4	10 (25)	4 (40)	6 (60)		1 (10)	4 (40)	4 (40)	1 (10)		2 (20)	7 (70)	

Table 2 The correlation between HPV16/18-E6 and p16 protein expression in cervical squamous cell carcinoma and the clinicopathological parameters

		HPV16/18E6 Expression			p16 Expression							
						Intensity				Dispersion		
Samples	Total	− (%)	+(%)	p	− (%)	weak (%)	moderate (%)	Strong (%)	p	Focal (%)	Diffuse (%)	p
SCC	40	19 (47.5)	21 (52.5)		8 (20)	10 (25)	17 (42.5)	5 (12.5)		9 (22.5)	23 (57.5)	
1. Pathologic tumor size												
<2 cm	9 (22.5)	8 (88.9)	1 (11.1)	0.041 (S)	2 (22.2)	4 (44.4)	3 (33.4)	0 (0)	0.190	4 (44.4)	3 (33.4)	0.073
2–5 cm	21 (52.5)	8 (38.1)	13 (61.9)		5 (23.8)	4 (19)	8 (38.2)	4 (19)		4 (19)	12 (57.2)	
>5 cm	10 (25)	3 (30)	7 (70)		1 (10)	2 (20)	6 (60)	1 (10)		1 (10)	8 (80)	
2. Histologic Grade												
WD	14 (35)	8 (57.1)	6 (42.9)	0.416	5 (35.7)	6 (42.9)	2 (14.3)	1 (7.1)	0.029 (S)	6 (50)	3 (21.4)	0.000 (HS)
MD	19 (47.5)	8 (42.1)	11 (57.9)		2 (10.5)	2 (10.5)	12 (63.2)	3 (15.8)		3 (10.5)	14 (79.8)	
PD	7 (17.5)	3 (42.9)	4 (57.1)		1 (14.3)	2 (28.6)	3 (42.8)	1 (14.3)		0 (0)	6 (85.7)	
3. Clinical stage												
I	17 (42.5)	10 (58.8)	7 (41.2)	0.521	4 (23.5)	7 (41.2)	6 (35.3)	0 (0)	0.035 (S)	6 (35.3)	7 (41.2)	0.037 (S)
II	11 (27.5)	4 (36.4)	7 (63.6)		2 (18.2)	3 (27.3)	5 (45.4)	1 (9.1)		3 (27.3)	6 (54.5)	
III/VI	12 (30)	5 (41.7)	7 (58.3)		2 (16.7)	0 (0)	6 (50)	4 (33.3)		0 (0)	10 (83.3)	
4. Lymph node metastasis												
No	28 (70)	15 (53.6)	13 (46.4)	0.344	7 (25)	9 (32.1)	11 (39.3)	1 (3.6)	0.007 (HS)	8 (28.6)	13 (46.4)	0.04 (S)
Yes	12 (30)	4 (33.3)	8 (66.7)		1 (8.3)	1 (8.3)	6 (50)	4 (33.3)		1 (8.3)	10 (83.3)	

HPV16/18-E6 Oncoprotein is Expressed in Lymphocytes and Endothelial Cells of Cervical Tumors and Adjacent Normal Tissues

To understand whether HPV-infected cervical tumors could spread to other organs through blood circulation, HPV16/18-E6 oncoprotein expression in normal cervical tissues adjacent to cervical carcinomas were examined by IHC. Our data showed that HPV16/18-E6 oncoprotein is indeed expressed in lymphocytes, macrophages infiltrating cervical tumors and adjacent normal cervical tissue and in endothelial cells of blood vessels (Figs. 1A–C). These results seem to support the possibility that HPV16/18 infection may spread to other organs through blood circulation.

The Cell Cycle Marker P16INK4a is Expressed in Cervical Squamous Cell Carcinoma

The p16 reaction was evaluated as positive when nuclear or cytoplasmic immunostaining was clearly demonstrated. Different patterns of p16 signal were observed based on staining intensity (weak, moderate and strong) and dispersion pattern (diffuse or focal). Thirty two cases (80 %) of the cervical cancer tissues were positive (Fig. 2). Moderate and diffuse pattern of p16 expression was statistically associated with histologic grade of the cervical cancer. Cases presenting a moderate p16 expression were more common in the advanced FIGO stages III/IV (50 %) while weak expression was associated with early stage I (41.2 %) ($p=0.035$) (Table 2). No association was observed between p16 staining intensity or dispersion and age of the patient, educational level, contraceptives administration, smoking status, number of pregnancy or tumor size ($p>0.05$) (Tables 1 and 2).

The Correlation Between HPV16/18-E6 and p16 Expression

The correlation between the expression of HPV16/18-E6 with p16 proteins intensity and dispersion in the cervical SCC is shown in Tables 3 and 4 respectively. The negative expression of p16 was significantly correlated with the negative expression of HPV16/18-E6 oncoprotein and the positive expression of p16 was significantly correlated with the positive expression of HPV16/18-E6 oncoprotein. These data were statistically significant ($p<0.05$).

Discussion

Cervical cancer remains the second most common malignancy in women in the world and accounts for 9.8 % of all cancer cases [31]. Infection with Human Papillomavirus (HPV) is considered to be the principal causative agent in the development of cervical squamous cell carcinoma [9]. The objectives of our study were to investigate the expression of HPV16/18-E6 and p16 in uterine cervical carcinomas in Qassim Region-Saudi Arabia, and to relate the results to the established

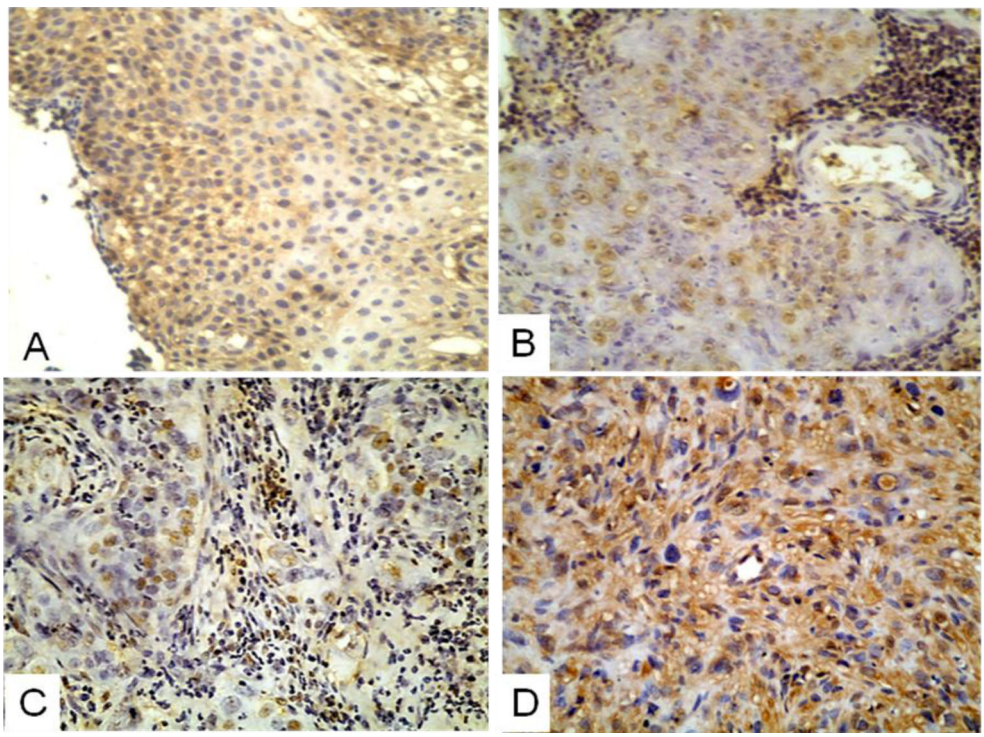

Fig. 2 Immunohistochemical analysis of p16 protein in cervical SCC. **A**: moderate cytoplasmic immunohistochemical staining of p16 in carcinoma in situ; **B** nuclear expression of p16 in well differentiated SCC; **C** nuclear expression of p16 in moderately differentiated SCC. **D** both cytoplasmic and nuclear expression of p16 in poorly differentiated SCC; the distribution of P16 expression was focal in **A**, **B** and **C** and diffuse in **D** (×400)

Table 3 The correlation between the expression of HPV16/18-E6 and p16 intensity in cervical squamous cell carcinoma

	HPV16/18-E6 Expression			
Samples	Total	Negative (%)	Positive (%)	p
Cervical SCC	40	19 (47.5)	21 (52.5)	
p16 Expression (intensity)				
Negative	8 (20)	7 (87.5)	1 (12.5)	0.000 (HS)
Weak	10 (25)	6 (60)	4 (40)	
Moderate	17 (42.5 %)	6 (32.3)	11 (64.7)	
Strong	5 (12.5)	0 (0)	5 (100)	

HS highly significant statistical correlation

clinicopathological prognostic parameters. A previous report showed the prevalence of HPV infection in invasive cervical cancer in Saudi Arabia (82 %) at the lower range of that observed in the world (85–95 %) [24]. Similar studies reported low prevalence of HPV infection among women with cervical cancer in Saudi Arabia and similar socio-cultural societies [14, 16, 20, 24].

Our data showed HPV16/18-E6 positivity in 21 cases (52.5 %); 73.6 % of positive cases were non Saudi (p <0.05). The non Saudi women showed development of cervical carcinoma at younger ages compared to Saudi women (p <0.05). A previous study demonstrated a trend for higher rates of HPV 16/18 infection leading to a greater proportion of high grade cervical lesion in young women in New Zealand [32]. A recent study observed the distribution of HPV16 variants worldwide and their relative risks for cervical cancer appear to be population-dependant [33].

The high-risk HPV types encode two oncoproteins, E6 and E7. Protein E6 is a transcriptional transactivator, binding double stranded DNA. It has transforming activity inactivating the human TP53/p53 tumor suppressor protein by targeting it for degradation [34]. The HPV E6 oncoprotein is involved in cell cycle deregulation, where p53 is abrogated. Sonoporation delivery of monoclonal antibodies against human papilloma virus 16 E6 restores p53 expression in transformed cervical carcinoma derived cell lines using chemical transfection [35]. The immunohistochemical expression of E6 has been proposed to be a useful maker for determining a diagnosis and/or prognosis of cancer [36]. The E7 oncoprotein binds to the retinoblastoma protein (pRb) tumor suppressor, leading to continuous cell cycling without any repair checkpoints [37]. In an attempt to prevent this continuous cell cycling, p16, a pRb regulator, is overexpressed and accumulates inside the cells [38].

Table 4 The correlation between the expression of HPV16/18-E6 and p16 dispersion in cervical squamous cell carcinoma

	HPV16/18-E6 Expression			
Samples	Total	Negative (%)	Positive (%)	p
Cervical SCC	40	19 (47.5)	21 (52.5)	
p16 Expression (dispersion)				
Negative	8 (40)	7 (87.5)	1 (12.5)	0.014 (S)
Focal	9 (25)	4 (44.4)	5 (55.6)	
Diffuse	23 (35)	8 (34.8)	15 (65.2)	

S significant statistical correlation

High-risk human papillomavirus E6 might contribute to some form of immunosuppression as it inhibits monocyte differentiation to Langerhans cells (competent antigen presenting cells) which in charge of the induction of T cell-dependent immunity [39, 40]. The vigilance of the immune system is readily exploited by HPV to escape immune destruction, resulting in persistent infections and development of HPV-positive cancers. HPV can evade host immune responses through avoidance of killing the host cells during viral replication and therefore neither presents viral antigen nor induces inflammation and downregulates the expression of HLA-class 1, facilitating evasion of cytotoxic T lymphocytes (CTL) attack [41]. Moreover, HPV16 E6 and E7 proteins reduce type-1 interferons (IFNs) expression in host cells thus inducing immune tolerance rather than the appropriate responses [41]. A recent study demonstrated that attenuation of IL-1β by the HPV16 E6 oncoprotein in immortalized cells is apparently a crucial step in viral immune evasion and initiation of malignancy [42].

Our data showed that HPV16/18-E6 oncoprotein is expressed in lymphocytes, macrophages, infiltrating cervical tumors and in endothelial cells of blood vessels. Previous studies demonstrated that peripheral blood mononuclear cells (PBMCs) might be HPV reservoir, thus spreading the virus through blood, providing a potential new route of transmission [43, 44]. Moreover a recent study demonstrated that HPV markers are not only found in PBMCs of peripheral blood but also present in CD20+ B lymphocytes and CD56+ natural killer cells in the semen of infected patients with high risk HPV-16 raising a concerns about the risk of developing cancers to distal organs and to the sexual partner [45]. On the other side, the competency of HPV to be uptaken by B lymphocytes cannot be excluded. In fact, this possibility is supported by the fact that heparan-sulfate proteoglycans, theorised to potentially be one of the primary receptors involved in HPV entry, are expressed by B lymphocytes and are a requirement for normal cell maturation, differentiation and function [46]. Although, sexual activity has been considered to be a major route of transmission for HPV resulting in genital cancers and oropharyngeal cancers [47], HPV infections in infants and female university students who are virgins, revealing that HPV transmission via other routes may exist [48, 49]. Furthermore, peripheral blood lymphocytes (PBLs) from healthy donors have been shown to be infected with HPV [44]. Therefore, we support the previous possibility that

HPV infection may spread to other organs through blood circulation [50, 51].

In the present study, immunohistochemical staining of p16 antibody in normal cervical epithelial tissues were negative. Meanwhile, 80 % (32/40 cases) of the invasive cervical cancer were positive for p16. A previous study observed p16 expression in 95.4 % of cervical squamous cell carcinoma [30]. Few studies have observed p16 expression in normal tissue [52, 53]. P16 is a protein that is expressed in low concentrations in healthy cells, but is overexpressed in cervical cancer and high-grade precursor lesions. Still, it is well established that non dysplastic epithelial cells can express p16, for example, under certain physiological conditions, such as the shortening of telomeres in older tissues. Here, the expression of p16 immediately induces cell cycle arrest and may ultimately induce apoptosis with the end results being a focal pattern with weak intensity [54]. Our study confirms increased expression of p16 in invasive cervical squamous cell carcinoma. Several studies have documented overexpression, with a diffuse and strong pattern, not only in high-grade cervical intraepithelial lesions but also with invasive cancer compared to the normal specimens [55].

The results obtained in our study showed p16 expression as predominantly in the nuclei of most of our tumor samples, while only a few of these tumors displayed both nuclear and cytoplasmic staining. Wang et al. and Murphy et al. found all the invasive squamous cancers exhibited strong nuclear and cytoplasmic staining. Thus, the p16 overexpressions either nuclear or cytoplasmic in affected cells are to be considered positive [56, 57]. The presence of p16 in the cytoplasm may results from a type of post transcriptional modification or overproduction of p16 protein which force its transfer to the cytoplasm [56].

In this study, we demonstrated a significant correlation between a diffuse and moderate p16 immunoreactivity, and high grade, stage, and lymph node metastasis of cervical SCC (P <0.05). A recent study observed that HPV16-E6 induces epithelial mesenchymal transition, thus may contribute to tumor progression and metastasis [58]. Previous studies observed strong and diffuse p16 expression in all cases of invasive cervical carcinoma [56, 57]. Consequently, p16 overexpression is a significant marker of cervical lesions and is considered to be a useful test that may facilitate an improved diagnosis of severe cervical lesions.

In our study, negative p16 and HPV16/18-E6 reactions were observed in combination in 17.5 % of cases, but 50 % of positive reactions were identified in combination. A correlation between p16 and HPV16/18-E6 expression was predicted, however, the reason for this discrepancy may be attributed to limitation of sensitivity of Immunohistochemical reaction [59, 51]. However, other risk factors or other types of high risk- HPV contribution such as HPV45 to the cervical carcinoma development could not be excluded. Theoretically, p16 represent a promising biomarker, because its expression reflects both that oncogenic HPV is present and that it has disrupted normal cell cycle function and might be useful diagnostic marker of oncogenic HPV infection. The increased level of p16 expression by high risk-HPV points to the recommendation of this protein as a marker of HPV infection [59–62]. However the detection of p16 per se, should not be considered as proof of high risk of HPV infection without consideration of other markers such as HPV16/18-E6. Our primary limitation is the relatively few number of cases of cervical carcinoma in Qassim region- Saudi Arabia, thus limiting adequate analysis.

Conclusions

This is the first study to investigate the expression of HPV16/18-E6 and p16 in uterine cervical squamous cell carcinomas in Qassim Region - Saudi Arabia. The present study revealed statistical correlation between HPV16/18-E6 expression in cervical carcinoma and nationality, smoking status and size of the tumor. HPV16/18-E6 oncoprotein expression in normal lymphocytes, macrophages, fibroblasts and endothelial cells in the tumor tissues and adjacent normal cervical tissues support the possibility that HPV infection may spread to other organs through blood circulation. P16 expression has been correlated with high grade, stage of cervical SCC and with HPV16/18-E6 expression. The current study supports the critical function of p16 and HPV16/18-E6 as specific markers for cervical carcinoma. However the potential for usage of p16 and HPV16/18-E6 as prognostic markers will require detailed follow data for a larger group of patients.

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics 2002. CA Cancer J Clin 55:74–108
2. Sankaranarayanan R, Madhukar A, Rajkumar R (2001) Effective screening programmes for cervical cancer in low- and middle-income developing countries. Bull World Health Organ 79(10): 954–962
3. GLOBOCAN 2000. Cancer incidence, mortality and prevalence worldwide, version 10. IARC Cancer Base No 5 Lyon, IARC Press. 2001
4. Bosch FX, de Sanjose S (2003) Human papillomavirus and cervical cancer – burden and assessment of causality. J Natl Cancer Inst Monogr 31:3–13
5. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Muñoz N (1999) Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189(1):12–19

6. de Sanjosé S, Diaz M, Castellsagué X, Clifford G, Bruni L, Muñoz N, Bosch FX (2007) Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis 7(7):453–459
7. Wright TC Jr, Schiffman M (2003) Adding a test for human papillomavirus DNA to cervical- cancer screening. N Engl J Med 348:489
8. What Causes Cancer of the Cervix?. American Cancer Society (2014-01-31).
9. Rashed MM, Bekele A (2011) The prevalence and pattern of HPV-16 immunostaining in uterine cervical carcinomas in ethiopian women: a pilot study. Pan Afr Med J 8:21
10. Missaoui N, Hmissa S, Frappart L, Trabelsi A, Ben Abdelkader A, Traore C, Mokni M, Yaacoubi MT, Korbi S (2006) p16INK4A overexpression and HPV infection in uterine cervix adenocarcinoma. Virchows Arch 448(5):597–603
11. Bhurgri Y, Bhurgri A, Rahim A, Bhutto K, Pinjani PK, Usman A, Hasan SH (1999) The pattern of malignancies in Karachi (1995 to 1996). J Pak Med Assoc 49:157–161
12. Altaf F (2001) Pattern of cervical smear cytology in western region of Saudi Arabia. Ann Saudi Med 21:94–96
13. Jamal A, Al-Maghrabi JA (2003) Profile of pap smear cytology in the Western region of Saudi Arabia. Saudi Med J 24:1225–1229
14. Raza SA, Franceschi S, Pallardy S, Malik FR, Avan BI, Zafar A, Ali SH, Pervez S, Serajuddaula S, Snijders PJ et al (2010) Human papillomavirus infection in women with and without cervical cancer in Karachi, Pakistan. Br J Cancer 102:1657–1660
15. Castellsague X, Bosch FX, Munoz N, Meijer CJ, Shah KV, de Sanjose S, Eluf-Neto J, Ngelangel CA, Chichareon S, Smith JS et al (2002) Male circumcision, penile human papillomavirus infection, and cervical cancer in female partners. N Engl J Med 346:1105–1112
16. Hammouda D, Clifford GM, Pallardy S, Ayyach G, Chekiri A, Boudrich A, Snijders PJ, van Kemenade FJ, Meijer CJ, Bouhadef A et al (2011) Human papillomavirus infection in a population-based sample of women in Algiers, Algeria. Int J Cancer 128:2224–2229
17. Alsbeih G, Ahmed R, Al-Harbi N, Venturina LA, Tulbah A, Balaraj K (2011) Prevalence and genotypes' distribution of human papillomavirus in invasive cervical cancer in Saudi Arabia. Gynecol Oncol 121:522–526
18. Al-Badawi IA, Al-Suwaine A, Al-Aker M, Asaad L, Alaidan A, Tulbah A, Fe Bohol M, Munkarah AR (2011) Detection and genotyping of human papiloma virus in cervical cancer specimens from Saudi patients. Int J Gynecol Cancer 21:907–910
19. Khorasanizadeh F, Hassanloo J, Khaksar N, Taheri SM, Marzaban M, Rashidi BH, Sari AA, Zendehdel K (2012) Epidemiology of cervical cancer and human papilloma virus infection among Iranian women - analyses of national data and systematic review of the literature. Gynecol Oncol 128:277–281
20. Hussain S, Bharadwaj M, Nasare V, Kumari M, Sharma S, Hedau S, Das BC (2012) Human papillomavirus infection among young adolescents in India: impact of vaccination. J Med Virol 84:298–305
21. Al-Muammar T, Al-Ahdal MN, Hassan A, Kessie G, Dela Cruz DM, Mohamed GE (2007) Human papilloma virus-16/18 cervical infection among women attending a family medical clinic in Riyadh. Ann Saudi Med 27:1–5
22. Duttagupta C, Sengupta S, Roy M, Sengupta D, Bhattacharya P, Laikangbam P, Roy S, Ghosh S, Das R (2004) Are Muslim women less susceptible to oncogenic human papillomavirus infection? a study from rural Eastern India. Int J Gynecol Cancer 14:293–303
23. de Boer MA, Vet JN, Aziz MF, Cornain S, Purwoto G, van den Akker BE, Dijkman A, Peters AA, Fleuren GJ (2006) Human papillomavirus type 18 and other risk factors for cervicacancer in Jakarta, Indonesia. Int J Gynecol Cancer 16:1809–1814
24. Alsbeih G, Al-Harbi N, El-Sebaie M, Al-Badawi I (2013) HPV prevalence and genetic predisposition to cervical cancer in Saudi Arabia. Infect Agent Cancer 8(1):15
25. Ren C, Cheng X, Lu B, Yang G. Activation of interleukin-6/signal transducer and activator of transcription 3 by human papillomavirus early proteins 6 induces fibroblast senescence to promote cervical tumourigenesis through autocrine and paracrine pathways in tumour microenvironment. Eur J Cancer. 2013 Aug 14.
26. Kaewprag J, Umnajvijit W, Ngamkham J, Ponglikitmongkol M (2013) HPV16 oncoproteins promote cervical cancer invasiveness by upregulating specific matrix metalloproteinases. PLoS One 8(8): e71611
27. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T (1998) Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. Am J Pathol 153(6):1741–1748
28. Bringold F, Serrano M (2000) Tumor suppressors and oncogenes in cellular senescence. Exp Gerontol 35(3):317–329
29. Lin HP, Wang YP, Chiang CP (2011) Expression of p53, MDM2, p21, heat shock protein 70, and HPV 16/18 E6 proteins in oral verrucous carcinoma and oral verrucous hyperplasia. Head Neck 33:334–340
30. Amaro-Filho SM, Golub JE, Nuovo GJ, Cunha CB, Levi JE, Villa LL, Andrade CV, Russomano FB, Tristão A, Pires A, Nicol AF (2013) A comparative analysis of clinical and molecular factors with the stage of cervical cancer in a Brazilian cohort. PLoS One 8(3): e57810
31. Mendoza L, Mongelos P, Paez M, Castro A, Rodriguez-Riveros I, Gimenez G, Araujo P, Echagüe G, Diaz V, Laspina F, Castro W, Jimenez R, Marecos R, Ever S, Deluca G, Picconi MA (2013) Human papillomavirus and other genital infections in indigenous women from Paraguay: a cross-sectional analytical study. BMC Infect Dis 13(1):531
32. Simonella LM, Lewis H, Smith M, Neal H, Bromhead C, Canfell K (2013) Type-specific oncogenic human papillomavirus infection in high grade cervical disease in New Zealand. BMC Infect Dis 13:114
33. Cornet I, Gheit T, Iannacone MR, Vignat J, Sylla BS, Del Mistro A, Franceschi S, Tommasino M, Clifford GM (2013) HPV16 genetic variation and the development of cervical cancer worldwide. Br J Cancer 108(1):240–244
34. Tungteakkhun SS, Duerksen-Hughes PJ (2008) Cellular binding partners of the human papillomavirus E6 protein. Arch Virol 153(3):397–408
35. Togtema M, Pichardo S, Jackson R, Lambert PF, Curiel L, Zehbe I (2012) Sonoporation delivery of monoclonal antibodies against human papillomavirus 16 E6 restores p53 expression in transformed cervical keratinocytes. PLoS One 7(11):e50730
36. Lin HP, Wang YP, Chiang CP (2011) Expression of p53, MDM2, p21, heat shock protein 70, and HPV 16/18 E6 proteins in oral verrucous carcinoma and oral verrucous hyperplasia. Head Neck 33(3):334–340
37. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, Stanley MA (2012) The biology and life-cycle of human papillomaviruses. Vaccine 30(Suppl 5):F55–F70
38. Cuschieri K, Wentzensen N (2008) Human papillomavirus mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia. Cancer Epidemiol Biomarkers Prev 17(10): 2536–2545
39. Iijima N, Goodwin EC, Dimaio D, Iwasaki A (2013) High-risk human papillomavirus E6 inhibits monocyte differentiation to Langerhans cells. Virology 444(1-2):257–262
40. Offringa RI, de Jong A, Toes RE, van der Burg SH, Melief CJ (2003) Interplay between human papillomaviruses and dendritic cells. Curr Top Microbiol Immunol 276:215–240
41. Sasagawa T, Takagi H, Makinoda S (2012) Immune responses against human papillomavirus (HPV) infection and evasion of host defense in cervical cancer. J Infect Chemother 18(6):807–815

42. Niebler M, Qian X, Höfler D, Kogosov V, Kaewprag J, Kaufmann AM, Ly R, Böhmer G, Zawatzky R, Rösl F, Rincon-Orozco B (2013) Post-translational control of IL-1β via the human papillomavirus type 16 E6 oncoprotein: a novel mechanism of innate immune escape mediated by the E3-ubiquitin ligase E6-AP and p53. PLoS Pathog 9(8):e1003536
43. Bodaghi S, Wood LV, Roby G, Ryder C, Steinberg SM, Zheng ZM (2005) Could human papilloma viruses be spread through blood? J Clin Microbiol 43(11):5428–5434
44. Chen AC, Keleher A, Kedda MA, Spurdle AB, McMillan NA, Antonsson A (2009) Human papillomavirus DNA detected in peripheral blood samples from healthy Australian male blood donors. J Med Virol 81(10):1792–1796
45. Foresta C, Bertoldo A, Garolla A, Pizzol D, Mason S, Lenzi A, De Toni L (2013) Human papillomavirus proteins are found in peripheral blood and semen Cd20+ and Cd56+ cells during Hpv-16 semen infection. BMC Infect Dis 13:593
46. Reijmers RM, Spaargaren M, Pals ST (2013) Heparan sulfate proteoglycans in the control of B cell development and the pathogenesis of multiple myeloma. FEBS J 280(10):2180–2193
47. Leemans CR, Braakhuis BJ, Brakenhoff RH (2011) The molecular biology of head and neck cancer. Nat Rev Cancer 11:9–22
48. Carozzi F, Gillio-Tos A, Confortini M, Del Mistro A, Sani C, De Marco L, Girlando S, Rosso S, Naldoni C, Dalla Palma P, Zorzi M, Giorgi-Rossi P, Segnan N, Cuzick J, Ronco G (2013) NTCC working group. risk of high-grade cervical intraepithelial neoplasia during follow-up in HPV-positive women according to baseline p16-INK4A results: a prospective analysis of a nested substudy of the NTCC randomised controlled trial. Lancet Oncol 14(2):168–176
49. Chiou HL, Wu MF, Liaw YC, Cheng YWSchiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S (2007) Human papillomavirus and cervical cancer. Lancet 370(9590):890–907
50. Chen TH, Huang CC, Yeh KT, Chang SH, Chang SW, Sung WW, Cheng YW, Lee H (2012) Human papilloma virus 16 E6 oncoprotein associated with p53 inactivation in colorectal cancer. World J Gastroenterol 18(30):4051–8.14
51. Roncaglia MT, Fregnani JH, Tacla M, DE Campos SG, Caiaffa HH, Ab'saber A, DA Motta EV, Alves VA, Baracat EC, Longatto Filho A (2013) Characterization of p16 and E6 HPV-related proteins in uterine cervix high-grade lesions of patients treated by conization with large loop excision. Oncol Lett 6(1):63–68
52. Giarnieri E, Mancini R, Pisani T, Alderisio M, Vecchione A (2000) Msh2, Mlh1, Fhit, p53, Bcl-2, and Bax expression in invasive and in situ squamous cell carcinoma of the uterine cervix. Clin Cancer Res 6(9):3600–3606
53. Volgareva G, Zavalishina L, Andreeva Y, Frank G, Krutikova E, Golovina D, Bliev A, Spitkovsky D, Ermilova V, Kisseljov F (2004) Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. BMC Cancer 4:58
54. Hampl M, Wentzensen N, Vinokurova S, von Knebel-Doeberitz M, Poremba C, Bender HG, Kueppers V (2007) Comprehensive analysis of 130 multicentric intraepithelial female lower genital tract lesions by HPV typing and p16 expression profile. J Cancer Res Clin Oncol 133(4):235–245
55. Mimica M, Tomić S, Kardum G, Hofman ID, Kaliterna V, Pejković L (2010) Ki-67 quantitative evaluation as a marker of cervical intraepithelial neoplasia and human papillomavirus infection. Int J Gynecol Cancer 20(1):116–119
56. Wang JL, Zheng BY, Li XD, Nokelainen K, Angström T, Lindström MS, Wallin KL (2005) p16INK4A and p14ARF expression pattern by immunohistochemistry in human papillomavirus-related cervical neoplasia. Mod Pathol 18(5):629–637
57. Murphy N, Ring M, Killalea AG et al (2003) p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. J Clin Pathol 56:56–63
58. Jung YS, Kato I, Kim HR (2013) A novel function of HPV16-E6/E7 in epithelial-mesenchymal transition. Biochem Biophys Res Commun 435(3):339–344
59. Qi ZL, Huo X, Xu XJ, Zhang B, Du MG, Yang HW, Zheng LK, Li J, Shen ZY (2006) Relationship between HPV16/18 E6 and 53, 21WAF1, MDM2, Ki67 and cyclin D1 expression in esophageal squamous cell carcinoma: comparative study by using tissue microarray technology. Exp Oncol 28(3):235–240
60. Galgano MT, Castle PE, Atkins KA, Brix WK, Nassau SR, Stoler MH (2010) Using biomarkers as objective standards in the diagnosis of cervical biopsies. Am J Surg Pathol 34(8):1077–1087
61. Nicol AF, Golub JE, e Silva JR, Cunha CB, Amaro-Filho SM, Oliveira NS, Menezes W, Andrade CV, Russomano F, Tristão A, Grinsztejn B, Friedman RK, Oliveira MP, Pires A, Nuovo GJ (2012) An evaluation of p16 (INK4a) expression in cervical intraepithelial neoplasia specimens, including women with HIV-1. Mem Inst Oswaldo Cruz 107(5):571–577
62. Cheah PL, Looi LM, Teoh KH, Mun KS, Nazarina AR (2012) p16 (INK4a) is a useful marker of human papillomavirus integration allowing risk stratification for cervical malignancies. Asian Pac J Cancer Prev 13(2):469–472