A STUDY ON HUMAN PAPILLOMAVIRUS USING IN SITU HYBRIDIZATION TECHNIQUE AND ITS ROLE IN CERVICAL NEOPLASIA

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Abstract

Background: clinical epidemiological studies have shown that human papillomaviruses play major role in the development of different types of cervical lesions, are therefore considered as the major infectious etiological agents of genital lesions and cancer.

***Objective*:** to determine the prevalence of HPV DNA by using in situ hybridization technique among archival tissue specimen of the uterine cervical lesions and normal cervical postmortem tissue biopsies.

***Material & Methods*:** Eighty cervical tissue samples were included in this study. 70 archival tissue biopsy samples comprised a risk group for HPV infection and / or cervical neoplasia; these were selected for the years from 1998 to 2005 from histopathology files of Al-kadhimiya teaching hospital, Al-Ilwiya teaching hospital, Al-Yarmouk hospital, Medical city department of teaching laboratories, and from four private laboratories. The patients mean age was 43.1 years with a range of 20 to85 years. The remaining 10 normal cervical postmortem tissue biopsies were obtained from the institute of forensic medicine and considered as control group. These autopsies were taken from virgin female cervices, their mean age 23.1 years with a range of 18-30 years. In Situ Hybridization was performed for the detection of HPV on cervical tissue.

Results: All normal control cases showed no specific signals for HPV DNA. 6 (30%) of 20 cases of cervical tissue with codylomatous changes, 1 (11.11%) of 9 cases of CIN I.3 (21.43%) of 14 cases of CIN II/III, and 9 (33.33%) of 27 cases of ISCC were shown to be positive for HPV 6/11/16/18/31/33 DNA.

Conclusion: The In situ hybridization enabling direct visualization of viral tissue distribution and better substantiate HPV as a causal agent in cervical neoplasia.A significant association ($p < 0.05$) was found between Insitu Hybridization signal pattern and the histological type of cervical noeplasia

Keywords: Human papilloma virus, cervical intra epithelial neoplasia, cervical carcinoma, in situ hybridization technique.

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Introduction

Various epidemiologic and studies have established a strong correlation between genital infection with human papilloma virus (HPV) and development of uterine cervix cancer (1).

This type of neoplasm represents approximately 6% of all human cancer and is the second most common form found in women (2).

The application in recent years of molecular biological techniques has resulted in the exponential growth of knowledge related to the biology and pathogenicity of HPV. To date, 78 different viral types causing human infection in the skin and mucosa are known (3,4). It has been found that 80 to 100 % of advanced neoplasm and

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invasive cervical carcinomas are caused by only few types of mucosotropic HPV. From the clinical point of view these have been classified as high {types 16 and 18}, intermediate {types 31, 33 and 35} or low {types6 and11} risk, and it is the type 16 that is the most prevalent in invasive carcinomas (30-60%). it has therefore been suggested that patients follow up and therapy should directed against the specific type of viral infection (5).

Since its introduction in 1969 by Gall and Pardue, in situ hybridization techniques have become important tools to detect nucleic acid target sequence(6).

This method has been widely used for detection of HPV nucleic acid .It easy to handle, reliable method for HPV detection and typing, working on both Pap smear and paraffin-embedded sections (7, 8).

The type of signal (cofluent, punctuate) may reflect either episomal or integrated forms of viral target DNA or the intensity of the signal HPV copy number. The main advantage of the in situ format is the ability to correlate DNA probe results with cellular morphology (9). Its main problem until now was its low sensitivity, with a detection limit of10 to50 copies per cell in formalin-fixed samples 7 therefore several attempts have been undertaken to enhance the sensitivity of ISH. Signal-amplified in situ technique have been developed to detect a small number of HPV nucleic sequences with high sensitivity by using a biotinylated probe for immunohistochemical detection of HPV in formalin-fixed, paraffin-embedded biopsy tissue sections. This assay is able to detect as few as 1 to 2 copies of target sequence per nucleus (10).

In premalignant lesions, the HPV genome is typically maintained in its episomal form. However, the majority of invasive cervical carcinomas contain HPV DNA that has integrated in to the host cell genome. Thus, the integration event is temporarily associated with the acquisition of malignant phenotype (11, 12, and 13). Integration has been shown in over 80% of HPV-16 positive SCCs and in 100% of HPV-18 positive SCCs (14).

Materials &Methods

Tissue samples: A total of 80-tissue sample from the uterine cervix were included in this study. 70 of 80 sample comprised a risk group in our study were obtained from archival paraffin embedded bocks selected for the years from 1998 to 2005 from histopathology files of Al- Kadhimya Teaching Hospital, Al-Ilwiya Hospital, Al-Yramouk Hospital, Medical city department of teaching laboratories, and from four private labarotories, These patients either had hysterectomy or punch biopsy, their mean age was 43.1 years with a range of 20 to85 years.

The remaining 10 samples (autopsies), which comprised a normal control group, were obtained from the institute of Forensic Medicine. These autopsies were taken from virgin female cervices; their mean age was 23.1 years with a range of 18-30 years, based on the fact that HPV infection is practically non-existent in celibate population (16).

Ethical approval for use of all specimens was obtained and the histopathological diagnosis was confirmed by review of freshly prepared hematoxylin and eosin-stained slides by certified pathologist and classified according to criteria outlined by the Word Health Organization (WHO).

Histopathologicaly, there were 10 cases of normal cervix, 20 cases of condylomatous changes (8 cases of mild,8 cases of moderate and 4 cases of sever), 23 cases of cervical intraepithelial neoplasia (9 cases CIN 1, 7 cases of CIN 2 ,and 7 of CIN 3) ,and 27 of squamous cell carcinoma of either the keratinizing or non keratinizing large-cell type (6 cases of well differentiated ,16 moderately differentiated, and 5 of poorly differentiated.

In Situ Hybridization for detection of HPV 6/11/16/18/31/33 DNA in Paraffin Embedded Sections:

Principle of the test: A biotinylated probe hybridized to the target sequence (HPV DNA), and then an anti-biotin antibody (Linker 1) binds to the biotin on the hybridized probe. In order to re introduce biotin into the system, an immunoglobulin (Linker 2) that is coupled with substantially higher numbers of biotin moieties than the probe binds to linker 1 antibody. These additional layers initiate amplification of the signal. To visualize the antibody/probe complex, streptavidin – alkaline phosphatase conjugate is added. Upon addition of the substrate BCIP/NBT (Bromo-chloro-indolyl-phosphate/Nitroblue Tetrazolium) solution, an intense blue signal appears at the specific site of the hybridized probe.

DNA probe Hybridization/Detection System In Situ Kit (Maxim Biotech, USA) was used.

The paraffin-embedded sections were cut into 4-6µm thicknesses, placed on Fisher brand positively charged slides and left over night to dry at room temperature.

The procedure of in situ hybridization was carried out in accordance with manufacturer's instructions. The process include a prehybridization steps for preparation of tissue slides, hybridization steps by placing 10-20µl of the ready use DNA probe/hybridization solution onto the tissue sections and the sections were covered with cover slips, then the slides were placed in the oven at 95Ċ for 10 minutes to denature the DNA. After that, the slides were put in the humid chamber and incubated at 37 Ċ overnight to allow hybridization of the probe with target nucleic acid. In the next morning, there are post hybridization steps for preparation of slides.

Slides were examine by light microscope and evaluated for integration status of HPV.A dot-like signal evenly distributed over most cell nuclei was interpreted as integrated HPV, and a patchy signal unevenly distributed over cell nuclei; was interpreted as episomal. Mixture of the two-signal pattern was interpreted as both integrated and episomal (mixed) [17].

Quality control:

Cervical tissue sections previously tested positive for HPV by PCR technique were used as positive control tissue.

Cervical tissue sections from virgin female autopsy were employed as negative control tissue.

Statistical Analysis

ANOVA analysis program was used to calculate the Mean, Standard deviation and difference in the mean of age among different histological types.

Statistical analysis by Chi-square – test was done to look for the relationships between the presence of HPV infection and the extent of histological

abnormality. All analysis was performed using SPSS program. A p value of less than 0.05 was considered statistically significant.

Results

Study population:

The study group consisted of 80 samples their age ranges from 18 to 85 years, with a mean of (40.61±13.668 S.D Years). The histological diagnosis was normal in 10 cases (12.5%), condylomatous in 20 cases (25.0%), CIN I in 9 cases (11.3%), CINII/III in 14 cases (17.5%) (Seven CIN II and seven CINIII) and invasive cancer (squamous cell carcinoma) in 27 cases (33.8%). Details of the subjects and their numbers, mean and the range of age are shown in (table 1).

There was highly significant difference in the mean of age between groups (p value< 0.01), and as shown in (table 2)

The mean age of control group (23.10±3.814 years) was significantly lower than the risk groups for HPV infection and /or cervical neoplasia (p<0.01).

Within the risk groups, the mean age of patients with condylomatous changes was significantly lower than ISCC (33.25±11.125 years Vs 49.67±10.126 years) (p <0.01). Whereas there was no significant difference between the mean age of patients with CIN I and the mean age of patients with CIN II/III or ISCC (42.00±9.605 years Vs 45.29±12.964 and 49.67±10.126 years, respectively) (p >0.05).Also there was no significant difference between the mean age of patients with CIN II/III and the mean age of patients with ISCC (45.29±12.964 Vs 49.67±10.126) (p>0.05).

Detection of HPV 6/11/16/18/31/33 DNAs in cervical lesions:

Detection of HPV6/11/16/18/31/33 DNAs was carried out on all specimens, and as shown in (table 3).

The In situ hybridization was considered satisfactory when (a) the endogenous positive control probe gave dark, even signal over almost every nucleus, indicating adequate digestion and availability of the target DNA, (b) the negative control probe gave no signal, (c) control section gave the appropriate reaction.

In (table 3), the Chi-square showed no significant association (p>0.05) between HPV infection and the extent of histological abnormalities. Although these results may give a rough idea about the frequency of HPV presence in different histological type of cervical lesions, they did not reach statistical significance because of limited number of cases tested and the using of probe that detected not only high risk but also detected high risk and intermediate risk HPV types (6/11/16/18/31/33, collectively).

Insitu hybridization signals in the nuclei showed various patterns but could be morphologically classified in to diffuse, dot, and mixed patterns. All normal cases showed no specific signals for HPV DNA. Of the condylomatous changes 6 of 20 cases (30%), displayed ISH signals for HPV DNA, and of these 6 cases, 5 showed diffuse patterns and only one case showed mixed pattern. In CIN I, only one of 9 cases (11.11%) was positive for HPV DNA and showed diffuse pattern. In CIN II/III, 3 of 14(21.43%) were positive for HPV DNA and showed diffuse, dot and mixed pattern respectively. Diffuse pattern was usually seen in the cells located from the middle to the superficial layer of the dysplastic epithelium, especially in the cells showing koilocytosis or marked

nuclear pleomorphism. While dot pattern tended to extend deep in to the parabasal and basal cells of the dysplastic epithelium. In ISCC, 9 of 27(33.33%) were positive for HPV DNA, and of these 9 cases, 3 showed dot signals and the remaining 6 cases showed mixed patterns. In general, diffuse Insitu hybridization signals were heterogeneous and focally distributed in both CIN and ISCC, whereas dot Insitu hybridization signals were entirely and evenly scattered on all of the cell nuclei as shown in (table 4).

It was evident from these data that dot or mixed Insitu hybridization signal were highest in the HPV positive CIN II/III and ISCC cases compared to condylomatous changes and CIN I .The Chi-square test showed that there was a significant association ($p<0.05$) between the Insitu hybridization signal pattern and the histologic types, and as shown in (table 5). The comparison between the observed and the expected values within CINII/III and the ISCC revealed a significantly higher integrated HPV (mixed or dot Insitu hybridization signals) compared to episomal HPV (diffuse Insitu hybridization signals).

Table 1: Age of the patients involved in the study

Lesions	Category	NO. (%)	Mean of age±S.D.*	Minus	Maximum
Normal	control	10(12.5)	23.1±3.814	18	30
Condylomatous changes	Case group1	20(25.0)	33.25±11.125	20	57
CIN I	Case group2	9(11.3)	42.00±9.605	26	60
CINII/CINIII	Case group3	14(17.5)	45.29±12.964	22	67
ISCC	Case group4	27(33.8)	49.67±10.126	35	85
Total		80(100)	40.61±13.668	18	85

* S.D.: standard deviation

Table 2: The difference in the mean of age among different histological types.

Histological type	Condylomatous changes	CIN I	CINII/III	ISCC
Normal	P < 0.05*	P <0.01**	P<0.01**	P <0.01**
Condylomatous changes		P >0.05	P >0.05	P <0.01**
CIN I			P >0.05	P >0.05
CIN II/CINIII				P>0.05
ISCC				

*Significant ** highly significant (ANOVA test).

Table 3: Number of HPV- positive and HPV- negative cases among different histological types of cervical tissue.*

Type	NO.	HPV Status			
		HPV-Positive		HPV-negative	
		NO.	%	NO.	%
Normal	10	0	0	10	100
Condylomatous changes	20	6	30	14	70
CIN I	9	1	11.11	8	88.89
CIN II/III	14	3	21.43	11	78.57
ISCC	27 Histological	9	33.33	18	66.67

*X^2 =5.751, P >0.05.

Table 4: Frequency and signals patterns of HPV 6/11/16/18/31/33 DNAs in relation to histological type of the cases examined

Histological type (NO.)	NO.OF HPV 6/11/16/18/31/33 DNAs positive (%)	Signal pattern		
		diffuse	dot	mixed
Normal(10)	0/10(0)	0	0	0
Condylomatous changes(20)	6/20(30)	5	0	1
CIN I(9)	1 /9 (11.11)	1	0	0
CIN II/III (14)	3/14 (21.43)	1	1	1
ISCC(27)	9 /27 (33.33)	0	3	6

Table 5: Association between HPV signal pattern and different histological types*

HPV6/11/16/18 /31/33 signal pattern	Observed/ Expected value	Histological type (NO.)					Total
		Normal	Condylomatous changes	CIN I	CIN II/III	ISCC	
Negative	O value	10	14	8	11	18	61
	E value	7.5	15.3	6.9	10.7	20.6	
Diffuse	O value	0	5	1	1	0	7
	E value	0.9	1.8	0.8	1.2	2.4	
Dot	O value	0	0	0	1	3	4
	E value	0.5	1.0	0.5	0.7	1.4	
Mixed	O value	0	1	0	1	6	8
	E value	1.0	2.0	0.9	1.4	2.7	
Total		10	20	9	14	27	80

*X^2 =21.381, P<0.05.

O= observed value, E= expected value

Discussion

In our retrospective study, it was not possible to evaluate the association between cervical lesions and different risk factors due to lack of clinical information. However, an obvious difference was noticed in the mean age of normal groups (23.10±3.814 years) was significantly lower than the risk groups (p, 0.01). This difference in age is likely attributed to the fact that the

females in the group with normal cervix were virgin and therefore were more likely to be young. While the mean age of patients with condylomatous changes was significantly lower than ISCC (33.25±11.125 years Vs 49.68±10.126 years) ($p<0.01$) and this follows the same epidemiological trend of the disease in developing countries that is a woman may become infected with HPV when she become sexually active and because persistent infection with high risk HPV is necessary for the development of CIN , the mean time between the initial infection and manifestation of invasive cervical cancer is estimated at about 15 years. Assessment of the minimal interval between HPV infection and manifestation of cervical cancer is currently the subject of many studies. This long development period suggest that, in addition to persistent infection with high risk HPV and immunological factors, the development of malignancy requires changes in the cellular genome of the HPV- infected cells 18.

There was no significant difference between the mean age of patients with CIN I and the mean age of patients with CIN II/III and ISCC (42.00±9.605 years Vs 45.29±12.964 and 49.67±10.126 years, respectively) ($P> 0.05$). In addition, there was no significant difference between the mean age of patients of patients with CINII/III and the mean age of patients with ISCC (45.29±12.964 years Vs 49.67±10,126 years) ($P >0.05$) and this consistent with a study suggest that approximately 15% of low grade, CIN I progress to high grade cervical lesion (CINII/III) within two years. Another study found that about one third of high-grade lesions (CINII/III) progress to cancer within ten years 8.

However, the peak incidence of cervical cancer in women more than 40 years old may be explained by the long duration of infection in older women infected with high-risk HPV genotypes. Moreover, persistent high-risk HPV infection in turn may increase the risk for the development and persistence of SILs 4. These facts should stress the need for early measures to detect these cervical lesions by cytological screening and hopefully prevent the development of cervical cancer 15.

Detection of HPV 6/11/16/18/31/33 DNAs in cervical lesions

In situ hybridization techniques are effective methods to detect and localize HPV DNA within the affected tissues. It is a reproducible, sensitive, specific, and precise method for localizing nucleic acid sequences in clinical tissue sections with exquisite preservation of tissue morphology. As an adjunct to conventional histopathology, Insitu hybridization may contribute to the understanding, diagnosis, and prognosis of disease 19.

The HPV DNA was detected by ISH with nonisotopic-labeled probe. The available probes for HPV detection consisted of a mixture of HPV probes 6/11/16/18/31/33. ISH for HPV DNAs was carried out on all 80 specimens. The results are summarized in table 3. All normal cases showed no specific signals for HPV DNA. Six (30%) of cases of cervical tissue with condylomatous changes, 1(11.11%) of nine cases of CIN I, 3 (21.43 %) of 14 cases of CIN II/III and 9 (33.33 %) of 27 cases of ISCC showed to be positive for HPV 6/11/16/18/31/33 DNA.

Our results were consistent with a molecular biology studies carried by Mohammed Ali (2002) in Iraq 20, who

found that 83.3 %, 33.3% and 24.24 % of archival tissues from patients with condylomata accumenata, preinvasive cervical neoplasia and ISCC, respectively were found to be HPV-DNA-PCR positive. Another study carried by Niakan et al (2003) in Iran 21 , showed that HPV DNA was detected in the nuclei of ISCC tumor cells in 13 (65%) of 20 cases using Insitu hybridization techniques.

Many of the studies were performed in non-Moslem western populations. The finding of these studies are often showed higher prevalence of HPV DNA in cervical lesions ranges from 25 to 90 %.HPV-16 accounts for the highest proportion, followed by HPV-18, HPV -45, and HPV- 31 , but their incidence varies depending on the country (22).

Our study was limited to the realistic possibility of determining significant clinical association between the extent of histological abnormality and the detection of HPV-DNA. From the overview of cervical cancer in Iraq we found that cancers of the cervix uteri constitute 1.4 % of the total number of cancers with annual number of 113 new cases of cervical cancer reported in 1995, 1996 and1997, respectively (Iraqi cancer board, 1999).

In this study, a significant association ($p < 0.05$) was found between Insitu hybridization signal pattern and the histological types, and as shown in (table 4) and (table 5) .Dot and mixed signal were detected among tissues with carcinoma and high grade CIN lesions but were rare among tissues with codylomatous changes (only one case showed mixed signal) and CIN I lesions, which characterized by diffuse signals occupying entire nuclei detectable in the mid/superficial layers, especially in the koilocytes, which are morphologically characteristics of HPV infection and probably indicate site of viral DNA replication in the superficial layers of the epithelium . This is consistent with the concept that papillomavirus replicates and undergo gene expression as a function of epithelial maturation (23).

In our study 1 of 3 HPV-positive high grade CIN showed increased from dot nuclear staining in the parabasal and intermediate layers to homogenous, strong nuclear staining in the superficial layers, confirming an increase in virus copy number, as has been reported previously by Stevenson et al. (2000) 24 . But HPV was not detected by the assay in basal/parabasal cells of CIN I lesions and cervical tissue with condylomatous changes.

The employment of in situ hybridization technique appears to be useful in detecting HPV infection within a histological test, and can be used as an indicator of HPV status. Our study showed that no significant association between the extent of histological abnormality and HPV infection. But there was a significant association between the Insitu hybridization signal pattern and the extent of histological abnormality. In all ISCC positive for HPV DNA, Insitu hybridization revealed a basic dot or mixed signal pattern in the nuclei, suggesting that HPV DNA integrated into tumor cell DNA. On the other hand, almost all codylomatous and CIN I displayed a diffuse, mainly epifocal, signal pattern.

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