Genotyping of Human Papilloma Virus Infections and Phenotyping of Tumor Infiltrating Lymphocytes in Iraqi Patients with Uterine Cervical Neoplasia
Majid Mohammed Mahmmod Al-jewari **, Saad Hasan Mohammed Ali**, Mohammed Khalifa Khudair Al-azzawi***

ABSTRACT:
BACKGROUND:
Among most basic and clinical investigations for detection and genotyping HPV, only In Situ Hybridization (ISH) is effective in studing the relation of HPV genome to that histopathological entity. Tumor Infiltrating Lymphocytes (TILs) represent the local cellular immune response against both cervical cancer and the associated HPV.
OBJECTIVE:
This study is aimed to investigate ISH in detection and genotyping of HPV and immunohistochemical (IHC) study in phenotyping of TILs in Iraqi women with different grades of cervical neoplasia.
MATERIALS AND METHODS:
This retrospective research included a total number of 129 cervical tissue blocks, 64 with invasive and 27 with pre-invasive cervical neoplasia, 12 with condylomata acuminata, 11 with chronic cervicitis and 15 with apparently healthy cervices.
Molecular detection and genotyping of HPV DNA were performed by using ISH whereas immunophenotyping was done by IHC using monoclonal antibodies for that specific CD markers representing some of TILs.
RESULTS:
The overall prevalence of HPV DNA in total group of invasive cervical neoplasia was 28.4%, 12 out of 48(25%) with squamous cell carcinoma (SCC) and 8 out of 16(50%) with adenocarcinoma (AC). HPV 16 constituted (58.3%) of HPV-positive invasive cervical SCC, where as HPV 31/33 and lastly HPV 18 constituted (25%) and (16.7%), respectively. In AC group, HPV 16, 18, 31/33 occupied 50%, 25%, 12.5% of this group, respectively.
The prevalence of HPV DNA in pre-invasive cervical neoplasia group was 22.2%. While no case with HPV 18 was detected, HPV 16 and HPV 31/33 genotypes occupied 40% and 60%, respectively.
Although CD3+, CD4+ and CD8+ lymphocytes showed high mean values, significant differences present only between HPV DNA-positive tissue blocks and control group (p<0.05). The CD20+ and CD56+ lymphocytes showed low mean values. Compared to control group, however, statistical analysis showed significant differences (p<0.05) regarding CD20+ and non-significant regarding CD56+ lymphocytes.
CONCLUSION:
Using ISH proved more powerful and sensitive tool in revealing precisely effects of HPV in cervical neoplasia compared to histopathological examination. High mean values of CD3+, CD4+ and CD8+ versus low mean values of CD20+ and CD56+ lymphocytes could reflect an important and specific role of these cellular responses against HPV viruses during initiation and progression of HPV-associated cervical cancers.
KEY WORDS: Human Papilloma Virus, Cervical Neoplasia, In Situ Hybridization, Tumor Infiltrating Lymphocytes, CD Markers.

* College of Science/Al-Mustansiyah University.
** *College of Medicine/Baghdad University.
*** College of Science / Diyala University

INTRODUCTION:
The rank of cervical cancer is on the top of the 10 most common female neoplasma in developing countries where as it is the fifth in western developed nations(1).
UTERINE CERVICAL NEOPLASIA

On reviewing cancer situation in Iraq, it was found that it constituted 1.4% of the total number of cancers (2).

During the past two decades, an overwhelming evidence was in favor of a causative relationship between infection with high-risk human papillomavirus (HPV) types and anogenital carcinogenesis (3,4).

World-wide, the massive toll of papillomavirus-associated cancer was 20% of all cancer death in women (5).

In addition, this virus is responsible for 500,000 new cases of cancer per year and billions of dollars in medical expenses (6).

Update, more than 120 HPV genotypes have been described and of these, 30 are causally related to different anogenital tumors and carcinomas (7).

For detection and typing HPV, most basic and clinical investigations use one or more of the following three nucleic acid-based tests: in situ hybridization (ISH); polymerase chain reaction (PCR); and hybrid capture system (HCS). However, only ISH is effective in studying the relation of HPV genome to that histopathological entity (8).

The development of successful cancer prevention strategies is facilitated by the understanding of the etiology of the disease, knowledge of factors that contribute to the carcinogenic process, and comprehensive immunological studies (9). The association between cancer and immune-suppression had long been recognized and considered to be fundamental event related to inception of the disease.

The presence of neoplasm is accompanied by distinctive alteration in the immune system, either manifestation of in situ anti-tumor response on one hand and depression of cell mediated immune function on the other (10).

Papilloma-specific antigens in cervical carcinoma are immunosuppressive or induce very poor cellular and humoral responses. It is not understood to what extent immunological factors contribute to the viral latency and the clinical manifestations of cervical cancer. However, Cigarette smoking, which is strongly associated with this disease, is associated with a decrease in cellular immunity and immunosuppressed patients are prone to develop viral genital lesions and change of cervical intraepithelial neoplasia (CIN) to cancer (11).

This research is also extended to study the tumor infiltrating lymphocytes that represent cellular immune status of those patients by using immunohistochemical study of a relevant panel of CD markers.

MATERIALS & METHODS:
This study is designed as retrospective research, where a total number of (129) formalin-fixed, paraffin-embedded cervical tissue blocks, belonging to patients who had undergone hysterectomy or a punch biopsy from the cervix, were included in this study.

They were collected from the archives of histopathology laboratories of different general hospitals as well as many private laboratories, including Teaching Laboratories and Specialized Surgeries Hospital in Medical City, Central Public Health Laboratories, Baquba Hospital Laboratories, Al-Yarmok Teaching Hospital Laboratories, Dr. Raji al-Hadihi private lab, Dr. Loay Edwar al-Khouri private lab, Dr. Ikbal Kobtan private lab and Dr. Naval Alash private lab.

Among 91 cervical neoplastic tissue blocks, there were 16 invasive adenocarcinoma, 48 invasive squamous cell carcinoma, and 27 different grades of cervical intraepithelial neoplasia (recent synonymous of dysplasia). This study also included 12 cervical tissues with condylomata acuminata, 11 cervical tissues with chronic cervicitis and 15 apparently healthy cervical tissues (i.e. without any significant pathological changes), obtained from hysterectomies of patients with uterine bleeding, were included as a diseased- and normal- control groups for this study, respectively.

Molecular detection and genotyping of HPV DNA in those tissue blocks was performed by advanced and recent generation of in situ hybridization (ISH), using a specific Biotinylted DNA probes for high-risk HPV genotypes including 16, 18, 31/33, whereas phenotyping of tumor infiltrating lymphocytes was done by immunohistochemical study of a panel of CD markers including CD3, CD4, CD8, CD20, and CD56.

Methodology:
Tissue sectioning and slide preparation:
In histopathological department at Teaching laboratories, each formalin-fixed paraffin-embedded block from each cervical biopsy was subjected to cut as serial thin sections of (5µm) thickness and were stuck on charge slides.

In order to prevent carry-over DNA contaminations from one tissue sample to another, only one
disposable cutting knife, that was specified for each tissue block, was used and then each section was stickered on a single charged slide. The 1st and 2nd tissue slides were specified for haematoxyline and eosin staining whereas many subsequent 5µm thickness-paraffinized tissue sections were specified for the following procedures of in situ hybridization As positive and negative HPV controls, it was feasible to include such tissue-containing charged slides in each test run by using cervical tissue blocks, proved by PCR to have both the cocktailed and high risk-oncogenic HPV genotypes, as a positive controls, as well as negative control tissue blocks, from those apparently healthy cervical tissues that were also proved by PCR technique to be negative for HPV.

In situ hybridization for a cocktailed - hpv genotypes:
1. Paraffin-embedded blocks of each biopsy were subjected to cut as serial thin section of thickness (5µm) and stickered on slide.
2. De waxing of paraffin by oven adjusted at 60°C for 30 minutes.
3. Slides with tissues are immersed sequentially in two changes of xylene for 5 minutes each.
4. Immersion in two changes of ethanol (99%) for one minute.
5. Immersion in three changes of ethanol (95%) for one minute.
6. Five rinses in distilled water.
7. Tissue digestion was done by immersion slides in target retrieval solution (Code No.S1699) (Dako corporation Co. Denmark) at 95-99°C for 40 minutes
8. Cooling the slide at room temperature for 20 minutes.
9. Four washes in deionized water or distilled water.
10. Immersion in target retrieval solution at 95-99°C for 40 minutes.
11. Cooling the slides at room temperature for 20 minutes.
12. Tissue rinsing by 4 washes in deionized water.
13. Immersion in 0.3% H₂O₂ in methanol for 20 minutes.
14. Tissue rinsing by 5 changes in deionized water.
15. Allowing the tissue on slide to be dried for 15 minutes.
16. Then 20µl of wide spectrum HPV probe that can be hybridized to many anogenital human papilloma viruses which include the following types: 6, 11, 16, 18, 31, 33, 35, 45, 51, and 52 (Code No. Y1404) (Dakocorp corporation Co., Denmark) was added to each slide.
17. Denaturation process of the HPV target DNA was done by placing the cover slipped-slide in pre-warmed oven at 92°C for 5 minutes.
18. Hybridization process is allowed to occur by transferring these slides to a pre-warmed humid chamber for hybridization to occur at 37°C for 60 minutes.
19. Removing of the cover slip is done by immersing these slides in tris-buffered saline with tween (20) (TBST) (Dakocorp corporation Co., Denmark) at room temperature for 5 minutes.
20. Again transferring those slides to a fresh TBST bath.
21. Immersion of these slides in stringent washing solution (Code No. K0601) (Dakocytomation Co., Denmark) at 48°C for 20-40 minutes in water bath.
22. Rising the slides in 3 changes of 1X TBST PH 7.6 for 1 minute.
23. Removing of the slides from TBST and carefully wiping the excess fluid present around the section.

Detection of hybridized probe:
1. Placing the slides on a level surface and completely covering the tissues by applying enough streptavidin-conjugated with alkaline phosphatase reagent (Code No.0601) (Dakocytomation Co., Denmark) to each section.
2. Incubation of 20 minutes at room temperature was then done.
3. Pouring off the streptavidin-AP reagent (code No.K0601) (Dakocytomation Co.Denmark) from each slide and immersing the slides in fresh TBST bath for 5 minutes was followed.
4. Removing the slides from TBST and carefully wiping the excess fluid around the section.
5. Placing the slides on level surface and completely covering the tissue by applying enough of 5-bromo-4chloro-3indoyl Phosphate/ nitro blue tetrazolium substrate-chromogen solution (BCIP/ NBT) (Code No. K0601) to each section.
6. Incubating the slides at room temperature for 1 hour.
Pouring off the substrate solution from each slide and immersing the slides in distilled water bath for 5 minutes.

After that immersing the slides in nuclear fast red stain (BDH Chemical Ltd., England) for 1-5 minutes. A standardization processes were done by using 1 minute, 2 minutes, 3 minutes, 4 minutes and 5 minutes immersion time for staining slides, then we found that the excellent results were obtained with 3 minutes incubation.

Washing process was followed by immersing the slides for 1 minute in distilled water.

ASSESSMENT OF THE RESULTS:
Within 2 hours, the obtained result was assessed by examining the processed slides under light microscope; a deposition of an soluble blue purple product at the sites of hybridization of the probes to their targets is a positive indicator for the presence of the questioned group of HPV.

In situ hybridization of hpy genotype 16, 18, 31/33 biotinylated dna probes:
The same procedures for in situ hybridization and detection of cocktailed probes of HPV (Dakocytomation Code No. K0601) were followed except for the step of the temperature for stringent washing of these steps was 58°C for 20-40 minutes.

Determination of Lymphocytes Phenotype by IHC technique
For the determination of the phenotype of some lymphocytes, 5 monoclonal antibodies for that specified CD markers, namely CD3,CD4,CD8,CD20,andCD56, were used.

Preparation of Slides and Staining
1. Serial thin sectioning of (4 µm) thickness was done for each paraffin-embedded biopsy block.
2. Sticking of the tissues sections was done.
3. Paraffin sections were deparaffinized in oven at 60°C for 30 minutes, then allowed for cooling.
4. The slides were then passed into 3 changes of xylene for 5 minutes each.
5. Placing the slides in a series of baths containing graded ethanol (100% to 80%) for 5 minutes each.
6. Transferring to distilled water bath was followed and kept for 5 minutes.
7. Washing in phosphate buffered saline, pH 7.6 for 10 minutes.

8. Enzyme digestion of the slides were done by treating with target retrieval solution (Dakocorporation Code no. 1699) at 95°C for 10-20 minutes.
9. Three serial washes were done in magnetic stirred PBS.
10. Drops of H2O2 0.3% in methanol were applied on the tissue sections for 10 minutes at room temperature.
11. Diluted protein power blocking reagent, (10% in PBS) was added to tissue sections for 10 minutes at room temperature.
12. Tissue sections were treated with diluted unlabeled mouse primary antibody and incubated in moist chamber for 1 hour at room temperature.
13. The tissue sections were washed by rinsing in diluted PBS for 5 minutes.
14. Secondary antibody was applied on tissue section for 20 minutes at room temperature.
15. Tissue sections were followed by washing with diluted PBS.
16. Then, tissue sections were treated with 20% diluted sterptavidin for 10 minutes at room temperature.
17. Tissue sections were treated (stained) with diluted liquid DAB for 30 minutes at room temperature.
18. Then washed with tap water.
19. Counter staining with hematoxyline/ eosin was done.
20. Washing with tap water.
21. Tissue sections were then dehydrated by a serial 80%, 90% ethanol for 5 minutes each.
22. Mounting with mounting medium and examined under light microscope.
23. Cell counting was performed by counting positively stained cells in 5 consecutive areas using a high power objective lens and ocular grid (40X).

Statistical Analysis
T test and ANOVA test, were applied to statistical examination of all results optioned in our research experiments.

RESULTS:
1 - Invasive cervical carcinoma group.
Figure 1 and 2 are showing positive- and negative-ISH reaction for cocktailed HPV DNA detection, respectively.
UTERINE CERVICAL NEOPLASIA

A– Squamous Cell Carcinoma: The total prevalence of HPV DNA in cervical squamous cell carcinoma tissue blocks was 33.3% the prevalence of HPV genotypes 16, 31/33 and 18 constituted 58.3%, 25% and 16.7%, respectively. Table 1,2 show the prevalence and genotypes of HPV in the group of cervical squamous cell carcinoma.

Table 1: Percentage of ISH detection of HPV DNA in the tissue blocks of cervical squamous cell carcinoma.

<table>
<thead>
<tr>
<th>Differentiation/grades of cervical squamous cell carcinoma</th>
<th>No. tested</th>
<th>% of total</th>
<th>HPV DNA detection</th>
<th>State of ISH</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiation</td>
<td>4</td>
<td>8.3</td>
<td>Positive ISH</td>
<td>2</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative ISH</td>
<td>2</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Moderate differentiation</td>
<td>27</td>
<td>56.3</td>
<td>Positive ISH</td>
<td>8</td>
<td>29.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative ISH</td>
<td>19</td>
<td>70.4</td>
<td></td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>17</td>
<td>35.4</td>
<td>Positive ISH</td>
<td>2</td>
<td>11.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative ISH</td>
<td>15</td>
<td>88.2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>100</td>
<td>Positive ISH</td>
<td>12</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative ISH</td>
<td>36</td>
<td>66.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: HPV - genotyping results of HPV – positive cervical squamous cell carcinoma.

<table>
<thead>
<tr>
<th>Squamous cell carcinoma differentiation</th>
<th>No. of HPV - positive tissue blocks</th>
<th>Cases showed this genotype</th>
<th>HPV genotype</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated</td>
<td>2</td>
<td>18</td>
<td>18</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>8</td>
<td>18</td>
<td>18+31/33</td>
<td>3</td>
<td>37.5</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>2</td>
<td>16</td>
<td>18+31/33</td>
<td>3</td>
<td>58.3</td>
</tr>
<tr>
<td>Any grade</td>
<td>12</td>
<td>18</td>
<td>18</td>
<td>2</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18+31/33</td>
<td>3</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Invasive cervical squamous cell carcinoma showing positive ISH reaction of HPV DNA (Cocktail) using BCIP/NBT substrate/chromogen and counter stained by nuclear fast red (x200).
B- Adenocarcinoma:
The total prevalence of HPV DNA in cervical adenocarcinoma tissue blocks was 50%. The HPV genotype 16 constituted 50%, HPV 18 constituted 25%, and HPV genotype 31/33 constituted 12.5%. Multiple (mixed) infection with HPV 16 and 18 constituted 12.5%. The overall prevalence of HPV DNA in total group of invasive cervical cancer was 28.4%. Table (3 and 4) show ISH detection and genotyping of HPV DNA in tissue blocks with adenocarcinoma.

<table>
<thead>
<tr>
<th>Table 3: Percentage of ISH detection of HPV DNA in tissue blocks with adenocarcinoma.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading / differentiation of adenocarcinoma</td>
</tr>
<tr>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>Well differentiated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Moderately differentiated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Figure 2: Invasive cervical squamous cell carcinoma showing negative ISH reaction of HPV DNA (Cocktail) using BCIP/NBT substrate/chromogen and counter stained by nuclear fast red (x200).
Table 4: Genotyping of HPV DNA in HPV-positive tissue blocks with adenocarcinoma.

<table>
<thead>
<tr>
<th>Adenocarcinoma differentiation / grading</th>
<th>HPV - positive tissue blocks</th>
<th>HPV genotype</th>
<th>No. of cases with this genotype</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiation</td>
<td>2</td>
<td>18</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31/33</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Moderate differentiation</td>
<td>5</td>
<td>16</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16+18</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>1</td>
<td>16</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16+18</td>
<td>1</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31/33</td>
<td>1</td>
<td>12.5</td>
</tr>
</tbody>
</table>

2- Pre-invasive cervical neoplasia group:
The overall prevalence of HPV DNA in this group was 22.2%. The cervical neoplasia tissue blocks that were associated with koilocytosis constituted 25.9%. But the prevalence of HPV DNA detection in tissue blocks with koilocytosis was 42.8%. The prevalence of HPV genotype 16 and 31/33 constituted 40% and 60% respectively. No HPV genotype 18 was detected in this group.

3- The control groups:
The groups of the tissue blocks that were apparently healthy and chronic cervicitis showed negative (ISH) results for HPV DNA.

4- Results of IHC for tumor infiltrating lymphocytes.
The mean values of CD3+, CD4+, CD8+, CD20+ and CD56+ T lymphocytes in HPV positive and HPV negative invasive and pre-invasive cervical neoplasia in comparison to the control group are shown in tables 5,6,7,8,9, respectively. The results of CD3+, CD4+, CD8+, and CD56+ lymphocytes showed high mean value in all groups and subgroups. In respect to CD3+ lymphocytes, only there were significant differences between HPV DNA-positive and HPV DNA-negative tissue blocks in comparison with control groups. Whereas only HPV DNA-positive tissue blocks showed significant differences (p<0.05) regarding CD4+ and CD8+ lymphocytes results.
The results of CD20+ and CD56+ TILs showed low mean values in all groups and subgroups of cervical carcinoma and pre-invasive neoplasia when compared to control groups. The statistical analysis showed significant differences (p<0.05) regarding CD20+ lymphocytes results and non significant differences regarding CD56+ lymphocytes.

Table 5: Distribution of infiltrating CD3 lymphocytes in (HPV positive and negative) cervical neoplasia and condylomata acuminata in comparison to control groups (apparently healthy, chronic cervicitis)

<table>
<thead>
<tr>
<th>No.</th>
<th>Histopathological entities</th>
<th>No. of cases</th>
<th>Mean value and SD</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV positive- invasive cancer</td>
<td>20</td>
<td>62.0 ± 6.5</td>
<td>Significant</td>
</tr>
<tr>
<td>2</td>
<td>HPV negative- invasive cancer</td>
<td>44</td>
<td>59.6 ± 5.5</td>
<td>Significant</td>
</tr>
<tr>
<td>3</td>
<td>HPV positive- CIN</td>
<td>6</td>
<td>66.6 ± 1.9</td>
<td>Significant</td>
</tr>
<tr>
<td>4</td>
<td>HPV negative- CIN</td>
<td>21</td>
<td>65.5 ± 1.9</td>
<td>Significant</td>
</tr>
<tr>
<td>5</td>
<td>Apparently healthy</td>
<td>15</td>
<td>37.5 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>
Table 6: Distribution of infiltrating CD4 lymphocytes in (HPV positive and negative) cervical neoplasia and condylomata acuminata

<table>
<thead>
<tr>
<th>No.</th>
<th>Histopathological entities</th>
<th>No. of cases</th>
<th>Mean value and SD</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV positive- invasive cancer</td>
<td>20</td>
<td>35.2 ± 6.0</td>
<td>Non-Significant</td>
</tr>
<tr>
<td>2</td>
<td>HPV negative- invasive cancer</td>
<td>44</td>
<td>31.8 ± 8.0</td>
<td>Non-Significant</td>
</tr>
<tr>
<td>3</td>
<td>HPV positive-CIN</td>
<td>6</td>
<td>48 ± 7.5</td>
<td>Significant</td>
</tr>
<tr>
<td>4</td>
<td>HPV negative-CIN</td>
<td>21</td>
<td>34.3 ± 5.9</td>
<td>Non-Significant</td>
</tr>
<tr>
<td>5</td>
<td>Apparently healthy</td>
<td>15</td>
<td>39.5 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Distribution of infiltrating CD8 lymphocytes in (HPV positive and negative) cervical neoplasia and condylomata acuminata

<table>
<thead>
<tr>
<th>No.</th>
<th>Histopathological entities</th>
<th>No. of cases</th>
<th>Mean value and SD</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV positive- invasive cancer</td>
<td>20</td>
<td>31.1 ± 4.0</td>
<td>Significant</td>
</tr>
<tr>
<td>2</td>
<td>HPV negative- invasive cancer</td>
<td>44</td>
<td>31.3 ± 2.5</td>
<td>Non-Significant</td>
</tr>
<tr>
<td>3</td>
<td>HPV positive-CIN</td>
<td>6</td>
<td>31.6 ± 7.1</td>
<td>Significant</td>
</tr>
<tr>
<td>4</td>
<td>HPV negative-CIN</td>
<td>21</td>
<td>35.3 ± 6.1</td>
<td>Non-Significant</td>
</tr>
<tr>
<td>5</td>
<td>Apparently healthy</td>
<td>15</td>
<td>29.1 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 8: Distribution of infiltrating CD20+ lymphocytes in HPV- positive and -negative cervical neoplasia

<table>
<thead>
<tr>
<th>No.</th>
<th>Histopathological Entities</th>
<th>No. of cases</th>
<th>Mean values and SD</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV-positive in invasive cervical carcinoma</td>
<td>20</td>
<td>±8.3 ± 3.2</td>
<td>Non Significant</td>
</tr>
<tr>
<td>2</td>
<td>HPV-negative in invasive cervical carcinoma</td>
<td>44</td>
<td>10.5 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HPV- positive in pre-invasive cervical neoplasia</td>
<td>6</td>
<td>11.0 ± 4.8</td>
<td>Significant</td>
</tr>
<tr>
<td>4</td>
<td>HPV-negative in pre-invasive cervical neoplasia</td>
<td>21</td>
<td>13.3 ± 4.5</td>
<td>Non Significant</td>
</tr>
<tr>
<td>5</td>
<td>Healthy control</td>
<td>15</td>
<td>8.8 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

1. p>0.05 2. p<0.01

Table 9: Distribution of infiltrating CD56+ lymphocytes in HPV- positive and -negative cervical Neoplasia

<table>
<thead>
<tr>
<th>No.</th>
<th>Histopathological Entities</th>
<th>No. of cases</th>
<th>Mean values and SD</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV-positive in invasive cervical carcinoma</td>
<td>20</td>
<td>2.6 ± 0.9</td>
<td>Non Significant</td>
</tr>
<tr>
<td>2</td>
<td>HPV-negative in invasive cervical carcinoma</td>
<td>44</td>
<td>2.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HPV- positive in pre-invasive cervical neoplasia</td>
<td>6</td>
<td>3.5 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HPV-negative in pre-invasive cervical neoplasia</td>
<td>21</td>
<td>3.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Healthy control</td>
<td>15</td>
<td>5.5 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION:
1- Cervical squamous cell carcinoma.
The present results showed similar percentage of HPV detection(40%) to recent study result performed by (12) in Iraq. However the present results were also lower than world wide perversive studies that displayed a higher percentage of HPV DNA in cervical squamous cell carcinoma. In these studies, the reported prevalences were more than 95% (13,4).
The high percentage of HPV genotype 16 among squamous cell carcinoma tissue blocks was found in the results of present study. These results are consistent with the results obtained by (14,15).
The previous studies had shown that HPV 16 genotype can reach a much higher viral loads than other HPV genotypes and that this genotype is associated with an increased severity of underlying cervical disease (16,17). The presence of such multiple infection with different HPV genotypes especially high oncogenic risk genotypes tends to increase the severity of the associated cervical disease (18).

2- Cervical adenocarcinoma.
The results which were obtained from the present study are in agreement with the results (52%) obtained by (19). Yet these results are not in agreement with previous study performed by (20).
This different appears lower than most previous studies but this could be a reflection of low prevalence of HPV in our general population. This is supported by the fact that sexual multipartners are not common in our society.
The results of present study show that high oncogenic- risk HPV genotypes (HPV 16 , 18) constitute the majority of the identified HPV.
Therefore, these HPV genotypes could play an important causal role in these cases of cervical adenocarcinoma. These results are in agreement with previous studies performed by (19,21).

3- Pre-invasive neoplasia.
The results that are detected in present study are relatively lower than the results of many other studies, prevalence in earlier studies yielded a range of percentages from 15% to 90% HPV DNA positivity in the range of cervical lesions from minimal neoplasia (through all grades of neoplasia) to invasive cervical cancer.
This could be explained to be related to the difference in the sensitivity of the used HPV detection methods as well as the difference in the prevalence of HPV reported in their relevant populations (22).
The results in present study are similar to these previous studies (23,12) and could be related to the free sexual behaviors activities of relevant populations(24).
It was documented, that most HPV infection are transient and did not persist except in high-risk HPV genotypes group (25).
Our results show 2 different HPV genotypes, 16and31/33. The majority of HPV types observed in this study had corresponded to the well-known intermediate- and high-risk HPV genotypes (i.e 31/33,35,56,16 and 18). These results are in agreement with results obtained by (12,26).
It was shown that the determination of oncogenic HPV genotypes in precancerous lesions was used as an important early marker of cancer, since each specific HPV genotype has showed differences in its association with the cervical cancer (27).
World-wide studies had shown that these HPV's are generally of a restricted high–risk group (e.g HPV 16 , 18 , 45 , and 56) among the 35 HPV genotypes commonly found in the cervical cancers. Moreover, it was reported that a lower frequent degree than high–risk group, intermediate–risk HPV group (e.g 31/33 and 35) are associated with all grades of neoplasia as well as invasive carcinoma (28,15).

4. Immunophenotyping of Tumor Infiltrating Lymphocytes:
Host – tumor interaction is a complex relationship that has yet to be fully understood. It appears that several factors may play role in determination of this relationship, such as the microenvironment of tumor as well as type and degree of host response (29).
Cellular anti tumor effects or systems such as Tumor Infiltrating Lymphocytes (TILs) and cytotoxic T-Lymphocytes (CTL) are involved in immunoregulation and immunosurveillance mechanisms through recognition of tumor associated antigens, such as tumor transplantation antigen, after presentation with MHC molecules (30).
Tumor infiltrating lymphocytes in the cervical tissues of all studied groups of this research work were tested for the surface markers using specific monoclonal antibodies.
The results of distribution of CD3 in HPV – positive and HPV – negative subgroups of pre-invasive and invasive cervical neoplasia are in agreement with those reported by (31,32,33,34).
UTERINE CERVICAL NEOPLASIA

In addition, it was revealed also that the count of CD4 – T lymphocytes was higher in HPV – positive group of invasive carcinoma than that in HPV negative counterpart. The lower mean values of CD4 cells may be due to a suppression effect of CD8 cells population on CD4 T – lymphocytes (35). In addition, eradication of infiltrating CD4 T – lymphocytes may reflect a suppression effect of Th1 and Th2 subsets on each other (30).

The present study also revealed that the number of CD8 lymphocytes reduced as tumor progressed. Many studies had suggested that the CD8 lymphocytes decreased in invasive carcinoma group. The results in the present study are similar to those obtained by(36,37,38).

While they reflect the same deficient immune function in cancer by itself, where they recorded low mean values in cancerous patients compared to healthy control, the non-significant differences between positive and HPV negative cervical cancer could indicate a non-important role of such cells in HPV infection.

The results of CD20+ cells showed lower mean values in comparison to the values of CD3+ T-cells. The decrease in number of CD20+ cell may reflect loss of T-helper rather than intrinsic B-lymphocytes. The B-cells are found to be corresponding to low or normal when T-cell levels are low (39).

The results in this study also display significant differences (P<0.05) only between pre-invasive cervical neoplasia group and control group. These results are closely similar to the results of the studies which were performed by (40,41). There were high significant differences (P<0.05) between HPV positive pre-invasive cervical neoplasia and control group. These results support the results of (42, 43). These findings reflect that humoral immune responses were locally higher in HPV infection of cervical neoplasia.

The results showed that cells expressing CD56 in HPV positive and HPV negative infected cervical lesions are in general of lower mean values when they are compared with their healthy control group. Interestingly the present results indicate that the mean values of CD56 cells are decreasing with the progression of cervical neoplasia. NK cells have the ability to kill any cell that express low levels of class I MHC antigen. Tumor cells overcome this potential ability for lyses by expressing of just those specific MHC antigen that can engage inhibitory receptors, which can prevent NK-cells activation(42,44).

CONCLUSION:
The low prevalence of HPV DNA in invasive cervical carcinoma group of this study could be, in part, attributed to low prevalence of HPV infection in general population. Using ISH technique for detection of HPV is more powerful and sensitive tool in revealing the precise effects of HPV in cervical neoplasia when compared to histopathological examination. High mean values of CD4+ and CD8+ versus low mean values of CD20+ and CD56+ lymphocytes could reflect an important and specific role of these cellular responses against HPV viruses during initiation and progression of HPV-associated cervical cancers.

REFERENCES:


UTERINE CERVICAL NEOPLASIA


31. Woodworth, C.D; Simpson, S. Comparative lymphokine secretion by cultured normal human cervical keratinocytes papillomavirus-immortalized and carcinoma cell lines. 1993; 142, 1544-55.


36. Torres JM; Cabrera T; Concha A; Oliva MR; Ruiz-Cabello F; Garrido F HLA class I expression and HPV-16 sequences in premalignant and malignant lesions of the cervix. 1993; 41, 65-71 cells in squamous intraepithelial lesions of the uterine cervix.


