ASSOCIATION OF HPV16 VIRAL LOAD IN GENE L2 WITH CANCER STAGES AND DEMOGRAPHIC CHARACTERISTICS IN CERVICAL CANCER PATIENTS FROM DHI-QAR PROVINCE, IRAQ

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Background. Human papillomavirus (HPV) infection, especially high-risk HPV16, is a risk factor for cervical cancer. HPV16 genotype demographic variations may affect carcinogenicity. HPV16 mutations and HPV16 DNA concentration were examined in cervical cancer development in Dhi-Qar province, Iraq. Aim of the study. The study endeavors to elucidate the demographic profile and the correlation between high-risk human papillomavirus type 16 and the susceptibility to cervical cancer in Dhi-Qar Province, Iraq, alongside comprehending the genetics of the minor capsid protein L2. The findings of the research could potentially aid in the timely detection of cervical cancer and enhance the efficacy of measures aimed at preventing and managing cervical cancer.

Material and Methods. 93 cervical cancer patients and 60 healthy controls participated in a 2017-2020 case-control research. L2 gene amplification detected HPV. HPV16 DNA concentration and demographic variations were assessed in cervical cancer patients.

Results. HPV16 infected 65% of cervical cancer patients, with a substantially greater viral load (1043.25±8.50 IU/ml) than healthy persons (91.25±2.90). Cervical cancer was more common in women aged 43-52 (37%) and 32-42 (30%). HPV16 infections peaked in 2019 (78%) and 2020 (69%), with older women having less infections. HPV16 infections were greatest in cervical cancer stages IV (70%), III (68%), II (60%), and 0 (60%).

Conclusion. HPV16 infections are rising among young women in Dhi-Qar province, Iraq, and HPV16 DNA concentration is associated with cervical cancer. The data suggest demographic differences in HPV16 genotype development of cervical cancer.

Keywords: cervical cancer, viral load, cancer stages, L2 gene, qPCR


Introduction

Cervical cancer is a notable public health issue on a global scale, ranking as the second most prevalent form of cancer among women worldwide. As per the World Health Organization's (WHO) estimations, the number of reported deaths in 2012 was 8.2 million, and the rate has remained stagnant in recent years [1]. Viral infections are responsible for a notable proportion of human cancers, approximately 15-20 percent, during the multi-stage progression of malignant tumors. Certain viruses are known to play a significant role in this process. It has been established in the last twenty years that specific viruses play a substantial role in the development of human cancer. The correlation between cancer incidence and Human papillomavirus (HPV) has been established. HPV is a sexually transmitted virus, and a significant proportion of cervical cancer specimens (99.7%) have been found to contain high-risk HPV DNA [2-4]. There exist a total of 12 distinct types of HPV, namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, which have been identified as possessing oncogenic characteristics. HPV 16 is considered the most hazardous among the 13 primary high-risk genital HPV types and is prevalent, causing more than 50% of all cervical cancer cases globally [5]. The prevalence of HPV infection in women without cervical lesions is globally estimated to be 11-12%, with the highest concentrations observed in sub-Saharan Africa (24%), Eastern Europe (21%), and Latin America (21%) [6-8]. The age-specific prevalence of HPV in the Americas and Africa exhibits a high incidence among individuals under the age of 25, followed by a decline in prevalence among those over the age of 45. The elevated incidence of HPV tumor genotypes 18 and 16 can be attributed to the development of cancer, as well as the presence of carcinogenic lesions and the extent of HPV contamination [9]. The development of cervical cancer has been shown to involve persistent infection with oncogenic types of HPV as a biological intermediary. Prior research has successfully identified the diagnostic markers for the aftereffect of high-risk HPV infection [10]. The potential utility of HPV viral load as a marker for persistence and to distinguish between regressing intraepithelial neoplasia2 (CIN2) and CIN3 lesions has been suggested based on 2-3 consecutive measurements of the degree of CIN. This finding has implications for the management of these lesions [11]. Research has indicated that the heightened viral load of HPV can serve as an alternative measure for persistence, and its capacity to forecast the likelihood of squamous intraepithelial lesions (SIL) has been documented in literature [12]. The objective of the current investigation is to evaluate the potential of HPV16 viral load as a prognostic indicator for the advancement of cervical cancer in Dhi-Qar Province. This will be accomplished by means of qRT-PCR amplification of the minor capsid protein L2. The L2 protein, with a length of roughly 500 amino acids, serves a crucial function in the virion assembly process and is believed to possess a molecular weight of 55KDa [13]. The
study endeavors to elucidate the demographic profile and the correlation between high-risk human papillomavirus type 16 and the susceptibility to cervical cancer in Dhi-Qar Province, Iraq, alongside comprehending the genetics of the minor capsid protein L2. The findings of the research could potentially aid in the timely detection of cervical cancer and enhance the efficacy of measures aimed at preventing and managing cervical cancer.

**Material and Methods**

**Study design**

The study in question was a case-control investigation that took place over the course of three years, from 2017 to 2020. The research was carried out at two distinct locations: the Yanka Kupala State University of Grodno and the Al-Hussein Teaching Hospital Histopathological Unit in Dhi-Qar Province, Iraq. The hospital administration obtained the consent form from the patients, and the study adhered to the principles of Al-Hussein Teaching Hospital. The study participants provided written informed consent to participate in the research.

**Sample collection**

The Histopathology Laboratory at Al-Hussein Teaching Hospital in Dhi-Qar Province, Iraq, collected samples from cervical cancer tissue blocks stored between 2017 and 2020. A total of 93 samples were obtained. The samples were organized based on the patient's name and age, diagnosed histologically, and categorized by the stage of cancer progression. An additional cohort of 60 women who were in good health were recruited to serve as the control group. The study obtained approval from the institutional ethics committee of Al-Hussein Teaching Hospital, with a designated approval number of 66 on 16.03.2020.

**DNA extraction**

The extraction of genomic DNA from tissue samples embedded in paraffin blocks was carried out using the G-spinTM Total DNA Extraction Kit, following the protocol for fixed tissues as provided by the manufacturer. The concentration and purification of total DNA were conducted using a Nanodrop spectrophotometer (THERMO, USA) by measuring the absorbance at 260/280 nm.

**Conventional PCR and Quantitative Real-Time PCR (qRT-PCR)**

In this study, the HPV qPCR primers utilized for the minor capsid protein L2 gene were developed through reference to the NCBI-Genbank database sequence (MH777342.2). The primer sequences are as follows: Forward - TGAAAATCCCGCCTTTGAGC and Reverse - TGTGCTTTCAAGGTGTTTTCAC. The preparation of the PCR master mix was conducted in accordance with the manufacturer's guidelines, utilizing the RealMODTM Green SF 2X qPCR mix. The components of the mix were subjected to centrifugation at 3000 rpm for a duration of 3 minutes, utilizing the Exispin vortex centrifuge. The PCR tubes were subjected to centrifugation at 3000 rpm for a duration of 3 minutes using the Exispin vortex centrifuge. Subsequently, the T100 Thermal Cycler manufactured by BioRad USA was introduced and standard thermocycling conditions were employed. The PCR thermocycler parameters were executed using an initial denaturation at 95°C for 5 minutes for a single cycle, followed by denaturation at 95°C for 30 seconds for 35 cycles, annealing at 58°C for 35 cycles, extension at 72°C for 1 minute for 35 cycles, and a final extension at 72°C for 5 minutes for a single cycle. To confirm the presence of a 511 bp band, the PCR products were subjected to visualization on a 1% agarose gel that was stained with ethidium bromide and exposed to UV light.

**Table 1. – Thermocycling conditions for qRT-PCR amplification**

<table>
<thead>
<tr>
<th>PCR step</th>
<th>Temp.</th>
<th>Time</th>
<th>repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial activation</td>
<td>95ºC</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95ºC</td>
<td>15 sec.</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>60ºC</td>
<td>30 sec</td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis**

The statistical analysis was performed on the data acquired. An independent samples t-test was conducted. The Mann-Whitney U test was conducted to analyze the dependent variables. The statistical analysis was conducted using GraphPad Prism v8.0 software, Statistical Package for Social Sciences version 20, and Microsoft Excel 2010. Statistical significance was determined by considering only those results that exhibited a p-value of less than 0.05.

**Results**

**Demographic data of studied samples**

The present research centered on the examination of HPV16 in females afflicted with cervical carcinoma. The present study involved a cohort of 93 female patients diagnosed with cervical cancer between 2017 and 2020. The data presented in Figure 1A indicates that the majority of cases were observed in the years 2018 (36%) and 2019 (29%), whereas the lowest number of cases were recorded in 2017 (17%) and 2020 (18%). These discrepancies in case distribution across the years are further highlighted in Table 2. The study included patients aged between 32 and 78 years, with a mean age of 49.34±3.94. The control group consisted of 60
healthy individuals aged between 32 and 77 years, with a mean age of 45.71±4.61. Table 3 provides a summary of the characteristics of the study participants. On the contrary, the age distribution of patients was categorized into four distinct groups, as illustrated in Figure 1B. The findings revealed that the majority of females diagnosed with cervical cancer were within the age bracket of 43-52 years (37%), followed by those aged between 32-42 years (30%) and 63-78 years (20%).

**Level of HPV-16 L2 gene in studied samples**

The current investigation employed the SYBR green qRT-PCR technique to quantify the HPV16 L2 gene (as depicted in Figure 2). The findings revealed that out of 93 women diagnosed with cervical cancer, 60 (65%) exhibited positivity for HPV16, whereas only 5 (8%) of the healthy control group tested positive (as illustrated in Figure 3A). The viral load was determined based on the viral copy number, revealing a statistically significant difference (P = 0.001) in viral load levels between the case and control groups. The study findings indicate that cervical cancer patients exhibit a high HPV16 load, which ranges from 1.09×102 IU/ ml to 5.07× 103 IU/ ml, with a mean ± SD viral load of 1043.25 ± 8.50 IU/ ml. Conversely, healthy individuals exhibit a significantly lower viral load, ranging from 88 IU/ ml to 101 IU/ ml, with a mean ± SD viral load of 91.25 ± 2.90 IU/ ml, as presented in Table 4.

**HPV-16 viral load in studied samples**

Figure 3B illustrates the distribution of viral load among different age groups of patients. The findings indicate that females diagnosed with cervical cancer between the ages of 32-42 and 43-52 exhibit the highest mean of HPV 16 load. However, the mean of viral load was observed to be similar in the age groups of 53-62 and 63-78 years. The findings indicate that there were notable variations in the transmission of viral load among distinct age cohorts of patients (P=0.021, X2=9.77, DF=3). Additionally, the distribution of viral load based on stages of cervical cancer revealed statistically significant variations (P=0.001, X2=55.8, DF=4), with the highest mean (4022 IU/ml) of HPV16 load observed in females diagnosed with distant metastasis (stage III). The findings indicate a rise in HPV16 load among patients in cancer stages II and IV, with respective mean values of 3172 IU/ml and 3200 IU/ ml. Conversely, the lowest mean value of viral load (987 IU/ml) was observed in stage I of cervical cancer, as illustrated in Figure 3C.
Table 4. – Compared case-control viral load
Таблица 4. – Сравнение вирусной нагрузки случай-контроль

<table>
<thead>
<tr>
<th>Viral Load (IU/ml)</th>
<th>Case-control comparison</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case</td>
<td>Control</td>
</tr>
<tr>
<td>Range</td>
<td>1.09×10^2 – 5.07×10^3</td>
<td>88-101</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>1043.25±8.50</td>
<td>91.25±2.90</td>
</tr>
<tr>
<td>SE</td>
<td>1.097</td>
<td>0.374</td>
</tr>
<tr>
<td>N.</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

*Significant correlation (p<0.05)

advanced stages III (40%) and IV (32%), while a smaller proportion of cases were identified in stage 0 (Carcinoma in situ) and stage II (invasion of surrounding organs or tissue), accounting for only 5% and 11% of cases, respectively. The present findings indicate statistically significant variations (p<0.05) in the prevalence of cancer stage across different age cohorts, as presented in Table 5. Specifically, the majority of patients in age groups 1, 2, and 3 exhibited cancer in stage III (42%, 41%, and 39%, respectively), whereas a higher proportion of elderly patients in age group 4 had cancer in the stage of distant metastasis (47%).

Figure 4. – Distribution of cases according to cervical cancer stages
Рисунок 4. – Распределение случаев по стадиям рака шейки матки

Detection of HPV-16 DNA by PCR
The findings of the genetic screening conducted on HPV DNA through conventional PCR indicate that 60 (65%) of the cervical cancer cases analyzed were found to be infected with HPV. In contrast, only 5 (8%) of the healthy control group were found to be positive for HPV. Statistically significant differences (p<0.05) were observed in the frequency of HPV between the cases and control groups, as presented in Table 6.

Distribution of HPV-16 infection in a time line
The survey findings (Figure 5) indicate a higher prevalence of HPV16 infections in 2019 (78%) and 2020 (69%) compared to the lowest recorded infections in 2017 (47%) (p = 0.045). The prevalence of HPV16 infection is influenced by age cohorts, as evidenced by the findings indicating a higher incidence of infections among younger
women in age groups 1 and 2, with a subsequent decline in infection rates among older women in age groups 3 and 4 (p = 0.034). On the other hand, the distribution of HPV16 infections with respect to cervical cancer stages indicated that the greatest incidence of infections was observed in stage IV (70%), succeeded by stages III (68%), II (60%), and stage 0 (60%), whereas only 45% of stage I cases exhibited viral infection (p = 0.076).

**Discussion**

According to epidemiological research, the primary measure for preventing cervical cancer and reducing expenses is the implementation of early-stage screening [14]. According to the source, the most notable variants of HPV include 18, 16, and 52 [15]. Consistent with prior research, the current investigation revealed that 60 instances out of 93 cases of cervical cancer exhibit positivity for HPV16. The frequency of viral genotypes in a Mexican population was found to be influenced by geographical area, as reported in a conducted study. According to a study conducted in Jalisco, Aguascalientes, and Zacatecas, it was found that HPV 51 and 16 exhibit the highest prevalence rates [16]. According to a separate investigation carried out in Tlaxcala, the genotype with the highest prevalence was HPV 16 and 18 [17]. According to current data, it has been observed that females belonging to the age groups of 32-42 years and 43-52 years exhibit the highest HPV16 copy number. This finding is consistent with a previous study conducted in Iraq by Pity et al. (2019), which reported the highest prevalence of HPV positivity in the age group of 30-49 years [18]. The absence of a coordinated vaccine program in Iraq has resulted in an unusually high incidence of cervical HPV, as reported in reference [19]. Multiple research studies have demonstrated that the evaluation of viral load in cervical specimens for oncogenic forms of human papillomavirus (HPV), such as HPV16, is a suitable method for predicting the likelihood of recurrent infection and the risk of developing squamous intraepithelial lesions (SIL) and cervical cancer (CC) [20]. The relationship between the progression of cervical cancer and the viral load of HPV is a topic of debate within the academic community. The current investigation examined the distribution of viral load in relation to various stages of cervical cancer. The findings revealed statistically significant differences, with the highest mean HPV16 load (4022 IU/ml) detected in females who had progressed to distant cancer (stage III). Additionally, an increase in viral load was observed in patients with cancer stages IV and II (3200 IU/ml and 3172 IU/ml, respectively). Conversely, the lowest mean viral load (987 IU/ml) was observed in stage I of cervical cancer. The aforementioned observation has been corroborated by previous research, which has documented a correlation between elevated HPV load in cytological investigations and a heightened
sustainability to developing carcinoma in situ [21]. Furthermore, the present findings are consistent with those of Moberg et al. (2005), who reported a positive correlation between elevated viral load in cervical smears and an increased risk of developing cancer in the future [22]. The hypothesis posited by Peitsaro et al. (2002) is congruent with the current study's findings. Differences in the sampling process can lead to variations in the quantity of cells present in a sample, ultimately impacting the viral load measurement. The absence of a standard has created a significant obstacle to the utilization of viral load. Multiple research studies have demonstrated a positive correlation between an escalation in viral load and an increase in both the severity and likelihood of lesions [23]. The researchers Shen et al. employed micro-cutting technology to collect viral load data across various stages of cervical cancer. The study findings indicate that viral load may serve as a significant independent predictor in cases of high-grade disease. Several recent studies have utilized viral load as a triage strategy, either in combination with other methods or as a standalone approach, for HPV primary screening [24]. Duan et al. employed the BioPerfectus Multiplex Real-Time PCR assay (BMRT) to quantitatively assess the quantity of single-copy genes in their study. The present assay exhibits the ability to identify 14 subtypes of high-risk HPV and 7 subtypes of low and medium risk strains, while also providing a quantification of viral load per unit cell. The qRT-PCR-based method that targeted the HPV16 L2 gene demonstrated uniformity in the outcomes for viral load, SIL, and cancer grade, according to a study [25]. The prevalence and types of circulating HPV vary significantly across diverse populations and age groups within populations. The current study reveals an increase in HPV16 infections in Dhi-Qar Province, Iraq, particularly among young women, and its association with the progression of cervical cancer. This finding is consistent with previous research conducted in different regions of the world, which has demonstrated the prevalence of HPV16 among young individuals and its involvement in the development and progression of cervical cancer [26]. With the exception of Latin America and the Caribbean, where prevalence tends to increase later in life in a bimodal pattern, a global trend of declining age-specific prevalence has been observed across all regions. The incidence and severity of specific types of HPV may vary, with a greater prevalence observed among Aboriginal and black populations based on ethnic differences. However, the present investigation contradicts recent research conducted in developed nations, which has suggested a decline in the incidence of HPV16 infection in recent times. This could be attributed to the implementation of vaccination and surveillance systems in those countries, which have consequently resulted in reduced infection rates [22]. In 2019, Torres-Poveda and colleagues noted that developed nations have experienced a decrease in the occurrence and mortality rates of cervical cancer over the last four decades. This can be attributed in part to the implementation of organized cytological and vaccination programs. HPV vaccines have been found to be highly effective in reducing genotype and disease infections in women who do not have a history of new or existing HPV infections. Despite the promising introduction of the HPV vaccine program, the challenges associated with HPV prevention and the treatment of related diseases persist in many developing and underdeveloped countries worldwide. This issue remains a pressing concern [27]. The prevalence of HPV-16/18 was found to be comparable to the findings of a previous study conducted in Mexico, which is of note. Regrettably, obtaining assertions concerning commensurate infection burdens is arduous due to the distinct trials, albeit inferior to those observed in other Latin American populations [28]. The study documented patient-reported symptoms and colposcopy findings within positive and negative HPV classes, as well as risk factors associated with HPV and HIV infections. These risk factors included age variability, multiple sexual partners, history of hormone contraception, and smoking. The topic of interest is the relationship between HPV. The prevalence and mortality rates of cervical cancer exhibit significant geographic variation, despite the availability of robust screening procedures, thereby rendering it a noteworthy public health issue. Moreover, there exists a notable variation in the prevalence of age-specific HPV across diverse demographic groups, with two distinct peaks of HPV positivity observed in younger and older women. Numerous studies have been conducted globally on account of the diverse HPV genotypes, the epidemiology of HPV infection, and carcinogens. Nonetheless, there exist several countries where the incidence based on population has yet to be observed. The screening protocols for cervical cancer exhibit variation across different nations. It is anticipated that organized cervical screening initiatives will yield greater success compared to opportunistic screening programs. The utilization of screening services has been associated with a reduction in the incidence and mortality rates of cervical cancer, as evidenced by various studies [29]. The prevalence, rate, and dispersion of the HPV16 genotype exhibit a low incidence in Central and Eastern Europe. The epidemiology of HPV infection in Kazakhstan, a country in Central Asia, lacks available data. The existing literature on the subject is limited to a few publications in international peer-reviewed journals and numerous articles in local medical journals [30]. The prevalence of HPV infection in African countries such as Nigeria, Guinea, South Africa, and Kenya is higher compared to that of the European population. Specifically, the incidence rates in Nigeria, Guinea, South Africa, and Kenya are 26.3%, 47.9%, 41%, and 38.8-42.3%, respectively, as reported in a previous study [31]. In this study, a PCR-based protocol was utilized to investigate whether the viral load serves as a reliable indicator of HPV 16 burden.

**Conclusion**

The present study concludes that there has been a surge in HPV16 infections in Dhi-Qar Province/
Iraq in recent times, particularly among the female youth population. Furthermore, these infections have been found to be linked with the severity of cervical lesions. qRT-PCR has the potential to serve as a primary screening modality for females who test positive for HPV. However, further investigation through larger cohort studies is required to establish the significance of qRT-PCR in the development of cervical cancer. The data acquired from this study holds significant value in the advancement of molecular diagnostic tools, establishing a correlation between epidemiological data.

References


АССОЦИАЦИЯ ВИРУСНОЙ НАГРУЗКИ HPV16 В ГЕНЕ L2 СО СТАДИЯМИ РАКА И ДЕМОГРАФИЧЕСКИМИ ХАРАКТЕРИСТИКАМИ У ПАЦИЕНТОВ С РАКОМ ШЕЙКИ МАТКИ ИЗ ПРОВИНЦИИ ДИ-КАР, ИРАК

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Введение. Инфекция вируса папилломы человека (ВПЧ), особенно вызываемая ВПЧ высокого риска типа 16 (ВПЧ16), – фактор риска развития рака шейки матки. Демографические вариации генотипа ВПЧ16 могут влиять на канцерогенность. Мутации ВПЧ16 и концентрация ДНК ВПЧ16 были исследованы при развитии рака шейки матки в провинции Ди-Кар, Ирак.

Цель. В исследовании делается попытка выяснить демографический профиль и корреляцию между ВПЧ16 и восприимчивостью к раку шейки матки в провинции Ди-Кар, Ирак, а также понять генетику второстепенного капсидного белка L2. Результаты исследования потенциально могут помочь в своевременном выявлении рака шейки матки и повысить эффективность мер, направленных на профилактику и лечение.

Материал и методы. 93 пациента с раком шейки матки и 60 здоровых людей из контрольной группы приняли участие в исследовании случай-контроль 2017-2020 гг. Амплификация гена L2 выявила ВПЧ. Концентрация ДНК ВПЧ16 и демографические изменения были оценены у пациентов с раком шейки матки.

Результаты. ВПЧ16 обнаружилась у 65% пациентов с раком шейки матки с существенно большей вирусной нагрузкой (1043,25±8,50 МЕ/мл), чем у здоровых людей (91,25±2,90). Рак шейки матки чаще встречался у женщин 43-52 лет (37%) и 32-42 лет (30%). Пик инфекций ВПЧ16 пришелся на 2019 (78%) и 2020 (69%) гг., при этом у пожилых женщин было меньше инфекций. Инфекции ВПЧ16 наиболее выражены при раке шейки матки в IV (70%), III (68%), II (60%) и 0 (60%) стадиях.

Выводы. Инфекции ВПЧ16 растут среди молодых женщин в провинции Ди-Кар, Ирак, и концентрация ДНК ВПЧ16 связана с раком шейки матки. Данные свидетельствуют о демографических различиях в развитии рака шейки матки по генотипу ВПЧ16.

Ключевые слова: рак шейки матки, вирусная нагрузка, стадии рака, ген L2, концентрация ВПЧ16.


Конфликт интересов. Автор заявляет об отсутствии конфликта интересов.

Финансирование. Исследование проведено без спонсорской поддержки.

Соответствие принципам этики. Исследование одобрено локальным этическим комитетом.

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