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Phylogenetic Analysis of HPV16 Isolated from Women with Cervical Cancer Based on *L2* Gene Partial Sequence in the Province of Dhi-Qar, Iraq

Abduladheem Turki Jalil[1,2]*, Aleksandr Karevskiy[1]

[1]*Department of Ecology, Faculty of Biology and Ecology, Yanka Kupala State University of Grodno, Grodno, Belarus;* [2]*Department of Medical Laboratories Techniques, Al-Mustaqbal University College, Babylon, Hilla, Iraq*

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Correspondence: Abduladheem Turki Jalil, Faculty of Biology and Ecology, Yanka Kupala State University of Grodno, Grodno, Belarus.
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Abstract

BACKGROUND: As a causative agent for cervical cancer, the human papillomavirus (HPV) is well-recognized. Being a high-risk form, HPV16 is considered and has been confirmed to be associated specifically with cancer of the cervix.

AIM: HPV16's phylogenetic tree and genetic diversity are well known worldwide, but in Iraq, documents on HPV16 genetic diversity among women with cervical cancer are lacking; therefore, the present study focused on phylogenetic analysis of HPV16 isolated cervical cancer based on L2 gene partial sequence.

METHODOLOGY: The current study focused on the investigation of HPV16 in women who suffer from cervical cancer. This survey was performed on 93 adult females suffering from cervical cancer during the period from 2017 to 2020. For the molecular study, DNA was extracted and subjected to a polymerase chain reaction (PCR) for detection and amplification of minor capsidprotein *L2* gene. Nucleotide sequences of amplified capsid *L2* gene were searched using the advanced simple local alignment search tool at GenBank (BLAST).

RESULTS: Genetic screening on HPV16 *L2* gene by conventional PCR showed that 60 (65%) of cervical cancer cases infected by HPV16 while only 5 (8%) of the healthy control group are positive for HPV16. The results of the NCBI-BLAST homology sequence showed that genetic variation appeared in four strains from five strains of HPV16.

CONCLUSION: HPV16 from the main causative factors for cervical cancer with high genetic variation.

Introduction

Internationally, it is the primary cause of death among women. Fifteen high-risk human papillomavirus (HPVs) are mostly considered to be caused by the prevalence of cervical cancer, based on recommendations established by the International Organization for Cancer Research (hrHPV). Due to their high carcinogenic potential, both the strains HPV16 and 18 are known as "high-risk [1], [2], [3]. Over 70% of low-grade intraepithelial cervical neoplasia (CIN), cellular alterations leading to high-grade CIN and invasive cervical cancer are caused by viral persistence of these two variations [4], [5], [6]. Besides, many other cancers, including prostate squamous cell carcinoma, cancer of the head and neck, and breast cancer have shown to be caused by the HPV16 [7], [8]. The HPV belongs to the family Papillomaviridae. The size of the viral genome varies from 7.9 to 8.1 kb in length, comprises of six early (E1, E2, E4, E5, E6, and E7) and two late (L1 and L2, respectively) genes [9], [10], [11]. The expression of L1 and L2 does not get sensed by the basal epithelial cells present in the nuclei of terminally differentiated cells [12], [13]. Empty virus like particles are generated by the self-assembly of the L1 and results in a range of recombinant expression systems that is the main concept present behind the approved HPV vaccines formulations (VLPs). These VLPs along with L1 are expressed and can be mixed with the L2. This study will focus on the L2 and its genetic variations. L2 plays an important role in the infectious processes and viral assembly [14]. L2 has a molecular mass of around 55KDa. However, in SDS-PAGE L2 exhibits an apparent molecular weight of 64-78kDa.Although post-translational L2 modifications have not been identified, the explanation for this incident remains uncertain. Rose *et al.* stated that L2 showed a doublet in native HPV virions, instead, it indicates that glycosylation was not a factor and that proteolytic cleavage may mean the form of lower molecular weight, but glycosylase therapy did not affect the size of L2. L2 has several core functional functions and multiple partners who interact. Deletion and/or mutagenesis studies have mapped the major domain sequences used by L2 [14], [15]. The study of the worldwide diversity of HPV variants is of significant importance. This variation in HPV sequence databases is helpful for epidemiological studies, for the production

of comprehensive diagnostic tests, and the successful designing of vaccines. Virus infectivity is known to be associated with relevant variants of the intertype HPV genome, pathogenicity, development of cervical cancer, assembly of viral particles, and host immune response. There is, however, no evidence yet to prove whether immunity from another version of one variant of HPV will defend against infection [16]. The present study focused to identify HPV16 genetic diversity throughout phylogenetic analysis of HPV16 isolated from women with cervical cancer based on *L2* gene partial sequence This may be important for the logical design of the diagnosis, medicinal, and vaccination interventions in particular clinical environments.

Materials and Methods

Sample collection

Samples were obtained from the histopathology laboratory at Al-Hussein Learning Hospital in Dhi-Qar province/Iraq, and 93 samples were taken from the blocks of cervical cancer tissues they had deposited in the period from 2017 to 2020. Samples were registered according to the patient's name and age, histologically diagnosed, and split according to the stage of the progression of cancer.

DNA extraction

Using the G-spinTM Complete DNA Extraction Package (fixed tissue protocol), genomic DNA was extracted from paraffin-embedded block tissue samples and carried out according to company guidelines. To ensure the purity of the DNA, we used a Nanodrop spectrophotometer (THERMO, USA) that measured the absorbance at (260/280 nm).

Polymerase chain reaction (PCR) reaction

In this analysis, HPV PCR primers (F:5'- CCGGCTACTGAAGT GGTGTT-3' and R: 5'-TACCAGCACGTTCAGCCAAT-3') for the main capsid protein L2 gene were designed using the NCBI-GenBank database sequence (MH777342.2). Using maxime PCR premix kit, the PCR master mix was prepared and this master mix was then applied to HPV16 DNA. According to company directions, all the PCR tubes were moved for 3 min at 3000 rpm to the Exispin vortex centrifuge. Put in the Thermocycler PCR then (T100 Thermal cycler. BioRad USA). Conditions of PCR thermocyclers using the standard form of PCR thermocyclers are shown in Table 1. To validate the existence of a 511 bp band, 1% agarose gel was run. The positive DNA bands were visualized under UV light after staining with ethidium bromide [5].

Table 1: Thermocycling conditions for PCR amplification

PCR strp	Temp (°C)	Time	Repeat
Initial denaturation	95	5 min	1
Denaturation	95	30 s	35 cycle
Annealing	58	30 s	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4	-	-

Statistical analysis

In addition to Microsoft Excel 2010, the computer program statistical application for social sciences version 20 (SSPS20) was used for further analysis. $p \leq 0.05$ was found statistically significant.

DNA sequencing method

The HPV16 DNA sequencing for samples was performed based on *capsid protein L2* gene, using agarose gel, The PCR product was purified by agarose gel. Then purified PCR products were sent to Macrogen Company in Korea for the sanger method to perform the DNA sequencing. In to get gene bank accession numbers, final genomic sequences were submitted to the GenBank-NCBI website, and alignment was made to show the phylogenetic analysis using the search tool (BLAST). Phylogenetic tree in was generated using the neighbor-joining method in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software [16], [17].

Results

Conventional PCR for detection HPV16 in cervical cancer

Throughout genetic screening on *HPV16 L2* gene by conventional PCR, present results showed 60 (65%) of cervical cancer cases infected by hrHPV16 while only 5 (8%) of healthy control group are positive for HPV 16 as shown in Table 2.

Table 2: Compared prevalence of HPV infection in cases and control

PCR detection of HPV	Cases	Healthy control	p-value
	n (%)	n (%)	
Positive	60 (65)	5 (8)	0.0031*
Negative	33 (35)	55 (92)	0.0111*
Total	93 (100)	60 (100)	

*Significant correlation ($p<0.05$).

The sequencing and phylogenetic tree of HPV

The results of sequence analysis of the capsid

protein *L2* gene for 5 samples showed the evolutionary relations between the studied strains with the nearest similar species of the HPV strains found in the gene bank data (Figure 1). GenBank number codes of these isolates are MW172583, MW172584, MW172585, MW172586, and MW172587 as shown in Table 3. The nucleotides sequences of all samples were processed using the BLAST alignment tool. The sequences were compared with the NCBI-GenBank data available based on the highest percentage or the lowest value. The ratio of similarity to the identified strains in the study with other strains from NCBI-GenBank ranged from 99 to 100%.

In the present phylogenetic tree, seven clusters (49, 51, 59, 65, 51, 5, and 60) were constructed in the MEGA 6.0 version using the Neighbor-Joining method. Local HPV isolates (HPV-1Q.1-IQ.5) are closed together and related to the type 16 isolate of NCBI-Blast HPV DL0098210 partial genome (MT316255.1)Complete Genetic Change (0.001–0.004%). NCBI-BLAST Identification of homology sequences between local HPV isolates and HPV isolates associated with NCBI-BLAST and showed that genetic variation appeared in four strains from five strains of HPV16 when the two mutations appeared in IQ-No.2 isolate (MW172584) at lociT/A and T/C, so three mutations identified in IQ-No.3 isolate (MW172585) at lociG/T, C/A, and T/A and one mutation detected in IQ-No.4 isolate (MW172586) at locus T/A. Moreover, two mutations were found in IQ-No.5 isolate (MW172587) at loci T/A and T/A as shown in Table 3.

Discussion

As well as the form most commonly associated with cancer, HPV-16 is the most common high-risk type in the world [18], [19]. The present results showed 60 (65%) of cervical cancer cases infected by hrHPV16 while the research of Pity and his friends in Duhok/Iraq. In 40.6% of cervical smears, HPV was identified. Hence, Pity *et al.*, remember that the absence of an organized vaccination program related to the unusually high prevalence of cervical HPV in Iraq [20]. Including Iraq and neighboring countries, this result falls in the middle of the broad range rates (9.8–86%) worldwide [21], [22]. HPV screening and its genetic variation may be a significant element in molecular diagnosis and vaccine methods in clinical settings. [23]. Numerous research groups worldwide have documented and published genetic diversity of HPVs along with the prevalence rate, especially high-risk HPV16 and HPV18 [5], [24], [25]. The phylogenetic study of preformed hrHPV16 relies on the genetic variations of early and late genes. In the present study, phylogenetic sequences of hrHPV16 were done depend on genetic analysis of minor capsid

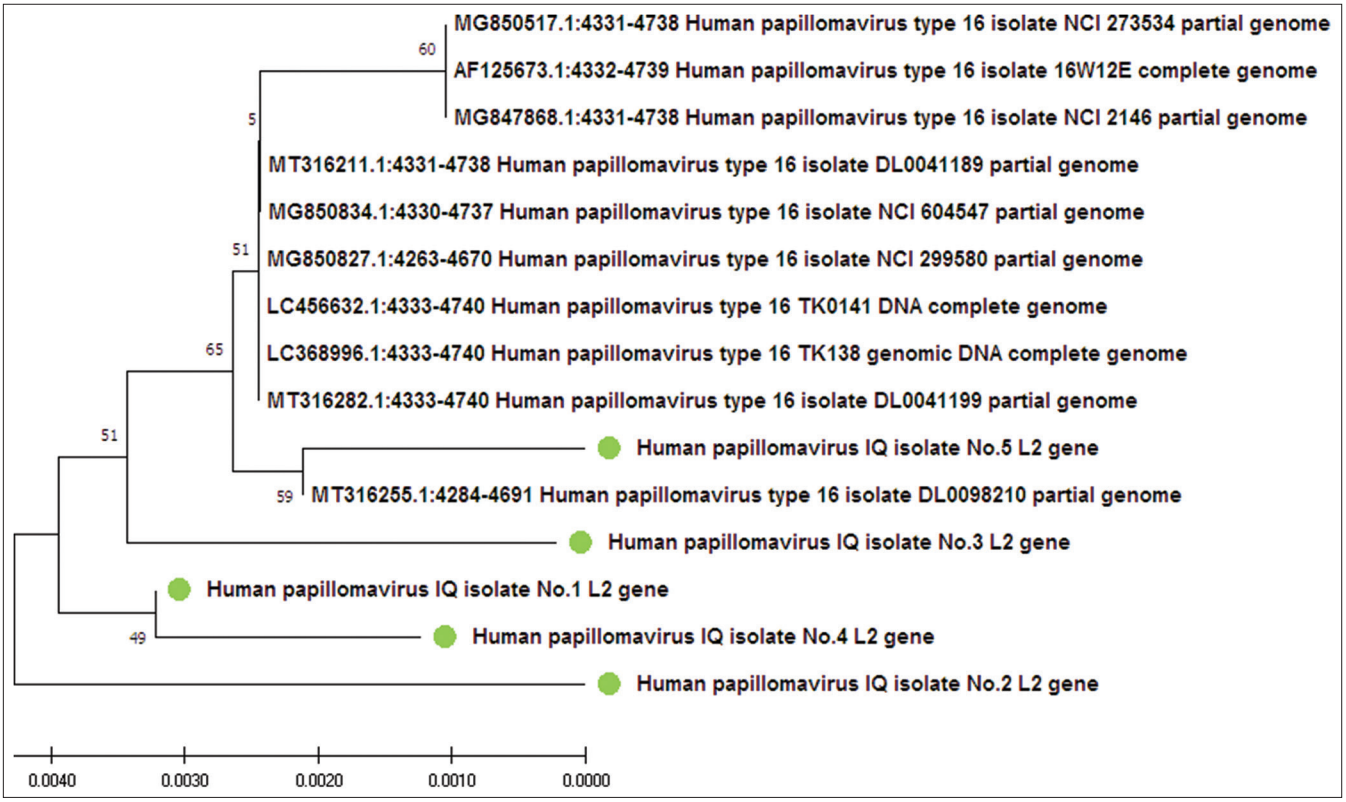

Figure 1: Partial sequence of phylogenetic tree analysis based on the capsid protein (L2) gene that was used for genetic analysis of HPV. Using the BLAST alignment tool, the nucleotide sequences of all samples were processed and the phylogenetic tree was constructed using evolutionary history using the Neighbor-Joining method (MEGA 6.0 version)

Table 3: NCBI-BLAST Homology sequence identification

Local HPV isolates	GenBank accession number	Numbers of Mutation	Alignment sequence size (bp)	Mutation %	Homology sequence identity (100%)	Mutation sites
Human papillomavirus IQ-No. 1 isolate	MW172583	0	396	0%	100%	Non
Human papillomavirus IQ-No. 2 isolate	MW172584	2	379	0.79%	99.21%	T/A, T/C
Human papillomavirus IQ-No. 3 isolate	MW172585	3	404	0.64%	99.26%	G/T, C/A, T/A
Human papillomavirus IQ-No. 4 isolate	MW172586	1	396	0.25%	99.75%	T/A
Human papillomavirus IQ-No. 5 isolate	MW172587	2	397	0.5%	99.50%	T/A, T/A
Range		0 – 3	379 – 404	0% – 0.79%	99.21% – 100%	
Mean ± SD		1.6 ± 1.14	394.4 ± 9.24	43.6% ± 31.45%	99.544% ± 0.33%	

protein L2, and to the best of our knowledge, there are very few studies about the hrHPV16 L2 gene worldwide, and this study the first in Iraq. However, the NCBI-Gen Bank database for nucleotide sequences of hrHPV16 L2 gene for current data showed that genetic variation appeared in four strains from five strains. The strains that coded MW172587 have high similarity (99%) with MT316255 and MT316282 previously recorded stains whereas the strains that coded MW172583, MW172584, MW172585, and MW172586 closely related together and have high similarity (99%) with MT316255 strain, previous research. On the other hand, In Uruguayan women, the HPV16 E6, E7 genes, and LCR were analyzed and a high variance in the LCR sequence was observed in 18 different strains. It was observed that novel non-synonymous substitutions in the E6 and E7 sequences showed primary lineage to the European variations. European and African clusters were also reported (6 strains) [26]. Genome wide association studies along with preclinical studies showed association of genetic variations in multiple risk loci. These locations were identified as the responsible for cervical cancer development [27], [28]. It was reported from the selective pressure study that maximum of the mutations present HPV-16 and HPV-58 L1 and L2 genes are positively associated with the HPV being revealed to adapt to its environment [29]. Substitution of nucleotides in viral genomes may have an effect on the assembly of viruses, carcinogenic potential, and host immunological responses [30]. The oncogenic potentials of these locations can be understood from the variety of HPV-16 genes. Moreover, it will also help to understand the effect of polymorphisms on the host response after infection or vaccination [29], [30].

Conclusion

In the presents study, the genetic variant of the HPV16 L2 gene was identified in the cervical cancer cases. This study reported some substantial data and a pragmatic approach to genetic diversity and phylogenetic analysis that in future could be very useful in correlating natural and evolutionary epidemiological studies with HPV. We believe this information generated about the HPV16 genetic variation will help Dhi-Qar province/Iraq to develop active molecular diagnostic systems, samples, and a next-generation prophylactic vaccination strategy that will, in turn, help the population of Iraq in eradicating this disease.

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