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Sequencing Analysis and Phylogenetic Tree of HPV Isolated from Breast Cancer Patients at Thi-Qar Province/Iraq

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***Abstract*— Genetic variety examination has demonstrated fundamental to the understanding of the epidemiological and developmental history of Papillomavirus (HPV), for the development of accurate diagnostic tests and for efficient vaccine design. The HPV nucleotide diversity has been investigated widely among high-risk HPV types. To make the nucleotide sequence of HPV and do the virus database in Thi-Qar province, and compare sequences of our isolates with previously described isolates from around the world and then draw its phylogenetic tree, this study done. A total of 6 breast formalin-fixed paraffin-embedded (FFPE) of the female patients were included in the study, divided as 4 FFPE malignant tumor and 2 FFPE of benign tumor. The PCR technique was implemented to detect the presence of HPV in breast tissue, and the real-time PCR used to determinant HPV genotypes, then determined a complete nucleotide sequence of HPV of L1 capsid gene, and draw its phylogenetic tree. The nucleotide sequencing finding detects a number of substitution mutation (SNPs) in (L1) gene, which have not been designated before, were identified once in this study population, and revealed that the HPV16 strains have the evolutionary relationship with the South African race, while, the HPV33 and HPV6 showing the evolutionary association with the North American and East Asian race, respectively.**

Keywords— HPV, Sequencing, Phylogenetic tree, Sanger, Breast Cancer words)

I. INTRODUCTION

The genome is characterized as the whole genetic data show in an organism. Genome sequencing is the methods to discover the actual configuration of nucleotides in the genome. Availability of the genome sequences provides the sequences of all the genes of organisms so that important genes influencing metabolism, cellular differentiation and development and disease process in animals, plants and microorganism can be identified and the relevant genes manipulated (Sangeeta et al., 2008). Genetic variety examination has demonstrated fundamental to a superior understanding of the epidemiological and developmental history of Papillomavirus (HPV), for the development of accurate diagnostic tests and for efficient vaccine design. The HPV nucleotide diversity has been investigated widely among high-risk HPV types (Latief et al., 2018). It was previously thought that all infectious wart-like lesions in humans were caused by a single type of HPV that affects different anatomical sites. After cloning more than 65 different HPVs, specific types of HPV were found to cause anatomically distinct infections (De Villiers, 1989). More than 200 HPVs, have been recognized and have been wholly sequenced (Doorbar et al., 2015). The HPVs can be characterized into low and high-risk types established on the risk that the virus will cause malignant tumor (IARC, 2007). A diverse HPV type is well-known when the DNA sequence of the L1 gene varies from that of any further categorized type by at least 10%. In addition, isolates of the same HPV type are mentioned to as variants or subtypes, when the nucleotide sequences diverge by lesser than 10% (Cavalcante et al., 2018). Each HPV type contains various genomic variations, and up to 2% nucleotide distinction has frequently been distinguished. This distinction presents every variation a naturally unmistakable portrayal and pathogenic dangers (Bernard et al., 2006).

This study aimed to make the complete nucleotide sequence of HPV DNA to do the virus database in Thi-Qar province, and compare sequences results of our study's isolates with previously described isolates from other region of the world and then draw its phylogenetic tree.

II. SUBJECTS AND METHODS

Patients: A total of 6 breast formalin-fixed paraffin-embedded (FFPE) tissue of the female patients were included in this study, 4 FFPE with malignant breast cancer and 2 FFPE of benign breast tumor. All of the samples were collected from Al-Hussein teaching hospital and Al-Haboby teaching hospital in Thi-Qar province/Iraq.Maintaining the Integrity of the Specifications.

Methods: The HPV DNA was extracted from FFPE tissue by using the gSYNC™ DNA Extraction Kit (Geneaid/USA). Estimation the concentration (ng/µl) and purity (260 /280 nm) of viral DNA will done by utilizing Nanodrop spectrophotometer (THERMO/USA). Then, utilizing the iNtRONs Maxime PCR PreMix Kit (I-Taq) to detect of HPV DNA by Conventional PCR. The products of PCR were analyzed by 1% agarose gel electrophoresis (Sambrook et al., 1989).

Primer		Sequence (5'-3')	Product Size
HPV-16 Primers	F	GTGGTAGATACTACACGCAGTAC	114bp
	R	ATATTCCTCCCCATGTCGTAGG	
HPV-16 probe	FAM-TGTGCTGCCATATCTACTTCAGAACCT-BHQ1		

The NEXpro™ qPCR Master Mix (Probe) and specific designed primer (viewed in following table) was used for detection of HPV16 genotypes based amplification of L1gene.

-Human Papillomavirus 16 Primers:

The analysis of Real-time data was performed by investigation of a threshold cycle number (Ct value) that exhibited the positive amplification in Real-Time PCR cycle number.

To substantiate genetic consistency and phylogenetic tree analysis of HPV16 types and to determine the genotype of the 2 unknown samples (after making HPV genotyping), the DNA sequencing method was done. The PCR product of HPV L1 gene to 6 positive samples (divided as 2 samples from malignant tumors, 2 samples from benign tumors and 2 unknown genotype samples from malignant tumors) were forward to Macrogen Company/ Korea to complete the DNA sequencing by the AB DNA sequencing system. The NCBI-BLAST Data analysis, Molecular Evolutionary Genetics Analysis version 7.0 (Mega 7.0) and Multiple sequence alignment analysis of the partial HPV L1 gene sequence based ClustalW alignment analysis were used to do the DNA sequencing analysis, and the evolutionary distances were registered utilizing the Maximum Composite Likelihood technique by phylogenetic tree UPGMA strategy.

III. RESULTS

The results of sequence analysis of the capsid protein (L1) gene for all samples, showed the evolutionary relations between the studied strains with the nearest similar species of the HPV genus found in the gene bank data. The nucleotide sequences of all samples were processed by using the FinchTV program to trim the base sequences. Then used the BLAST alignment tool. The sequences were compared with the data available in the NCBI-Genbank based on the highest percentage or lowest value. The ratio of similarity to the identified strains in the study with other strains from NCBI-Genbank ranged from 89-100% as in the fig. (1) and table (1).

The HPV16 malignant breast tumors IQ-No.1 isolate (MK388167.1) and HPV16 benign breast tumors IQ-No.4 isolate (MK388170.1) showed 100% match, while HPV16 malignant breast tumors IQ-No.2 isolate (MK388168.1) and HPV16 breast malignant tumors IQ-No.3 isolate (MK388169.1) showed a 99% match with (AY177679.1) HPV isolate isolated from South Africa.

Human papillomavirus type 6 benign breast tumors IQ-No.5 isolate (MK388171.1) revealed a 100% match ratio, with (AF335604.1) isolate isolating from China.

Human papillomavirus type 33 malignant breast tumors IQ-No.6 isolate (MK388172.1) revealed a 99% match ratio, with (GQ479019.1) isolate isolating from Canada.

The optimal tree was plotted for all studied isolates. The evolutionary distance was calculated using the basic differences of each sequence. MEGA7 was used to draw the evolutionary tree of each strain after comparing it with the ten closest strains of each line of the HPV genus, as shown in fig (2).

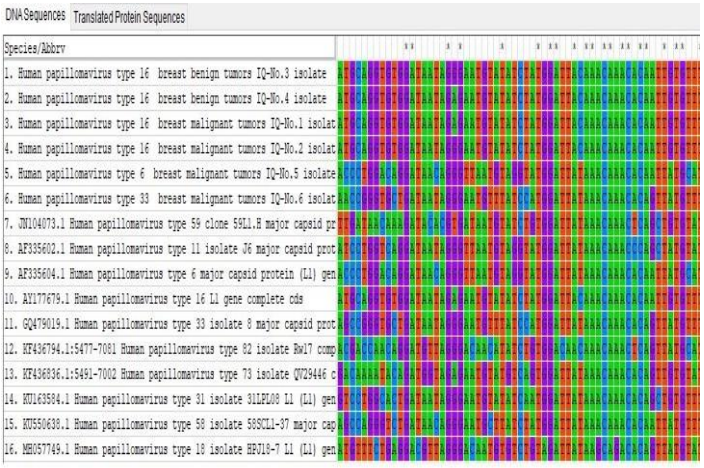

Figure (1):Multiple sequnce alignment analysis of the partial capsid protein (L1) gene squence in HPV genotypes isolates and NCBI-Genbank Human genotypes papillomavirus isolates by using ClustalW for (MEGA7.0, multiple alignment analysis tool) The multiple alignment analysis was shown the similarity (*) and substitution mutation in capsid protein (L1) gene nucleotide squences.

table(1):the NCBI-BLAST Homology sequence idenity between local HPV genotypes isolates and NCBI-BLAST related HPV genotypes.

Local HPV isolates	Local isolates an accession number	NCBI-BLAST Homology sequence identity			
		Identical NCBI-BLAST HPV Genotype	An accession number	Country	Identity (100%)
Human papillomavirus type 16 breast malignant tumors IQ-No.1 isolate	MK388167.1	NCBI-HPV-16	AY177679.1	South Africa	100%
Human papillomavirus type 16 breast malignant tumors IQ-No.2isolate	MK388168.1	NCBI-HPV-16	AY177679.1	South Africa	99%
Human papillomavirus type 16 breast malignant tumors IQ-No.3 isolate	MK388169.1	NCBI-HPV-16	AY177679.1	South Africa	99%
Human papillomavirus type 16 breast benign tumors IQ-No.4 isolate	MK388170.1	NCBI-HPV-16	AY177679.1	South Africa	100%
Human papillomavirus type 6 breast benign tumors IQ-No.5 isolate	MK388171.1	NCBI-HPV-6	AF335604.1	China	100%
Human papillomavirus type 33 breast malignant tumors IQ-No.6 isolate	MK388172.1	NCBI-HPV-33	GQ479019.1	Canada	99%

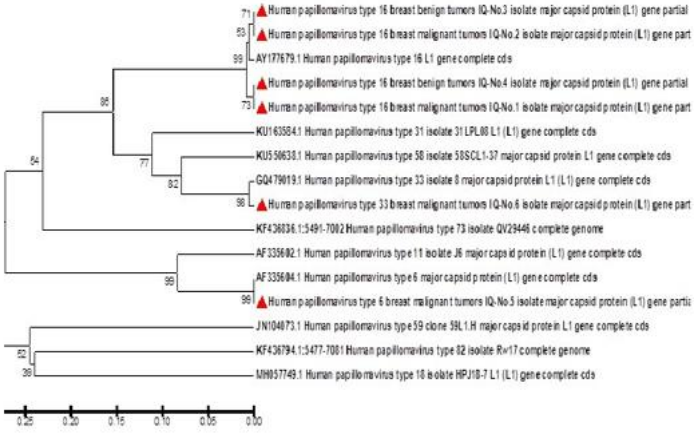

Figure (2): Phylogenetic tree analysis based on the capsid protein (L1) gene partial sequence that used for HPV genotyping detection. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 7.0 version). The local HPV isolates (HPV-1Q.1-IQ.4) were show closed related to NCBI-Blast HPV type33 (AY177679.1),the local HPV isolated (HPV-1Q.5) were show closed related to NCBI-Blast HPV type 6 (AF335604.1), and the local HPV isolates (HPV-1Q.6) were show closed related to NCBI-Blast HPV type 33 (GQ479019.1) at total genetic change (0.05-0.30%).

IV. Discussion

The sequencing analysis of nucleotides of worldwide strains of HPV16 and HPV-18 isolates indicates co-evolution of these genotypes with the three main ethnic categories: Africans, East Asians and Caucasians. In distinction to the phylogenetic tree of HPV 6, HPV 16 and HPV18 variants strongly separate into five most important variant ancestries: European, Asian, Asian-American, and two African lineages (Ho et al., 1993; Matos et al., 2013).

The dispersal of HPV-16 isolates worldwide diverges pointedly and associates with the essential admixture level of every resident.

The Human Papillomavirus 6 variations are distinguished with comparative frequencies around the globe (Heinzel et al., 1995).

Presented data on HPV 33 genomic variability is rare and only a limited HPV 33 genomic variation must be designated so far (Bokal et al., 2010).

The genomes sequenced in the present study were compared to the 10 HPV isolates designated previously and presently existing in NCBI Genbank. The present study adds 6 new sequences of HPV isolates in Thi-Qar and make our data base. The two isolates of HPV16 IQ-No.1 isolate (MK388167.1) and IQ-No.4 isolate (MK388170.1), which isolated from malignant breast tumors and benign breast tumors, respectively, showed 100% match with HPV Africaian isolate (AY177679.1) (Varsani et al., 2003), while two isolates of HPV16 from breast malignant tumors IQ-No.2 (MK388168.1) and HPV16 IQ-No.3 isolate (MK388169.1) showed a 99% match with the same isolate.

The Human Papillomavirus type 6 IQ-No.5 isolate (MK388171.1) which isolated from benign breast tumors, revealed a 100% match ratio, with (AF335604.1) isolate isolating from China (Mingce et al., 2000).

The sequence analysis results show a 99% match ratio between local isolated HPV type 33, which isolated from malignant breast tumors IQ-No.6 isolate (MK388172.1) and (GQ479019.1) isolate isolating from Canada (Cornut et al., 2009).

The finding achieved in this study detects a number of substitution mutation (SNPs) in capsid protein (L1) gene nucleotide sequences, which have not been designated before were identified once each in this study population.

The phylogenetic trees were assembled for the capsid protein (L1) gene nucleotide sequences of 16 HPVs, using MEGA7 analytical systems. The resulting phylogenetic trees exactly classified the HPVs into diverse clinically related types, including their previously described tissue tropism and high/low oncogenic activity.

V. Conclusion

The nucleotide sequencing detects a number of substitution mutation (SNPs) in L1 gene, which have not been designated before, were identified once in this study population. The HPV 16 strains showed the evolutionary relationship with the South African race, while the HPV 33 and HPV 6 exposed the evolutionary correlation with the North America and East Asia race, respectively.

VI. References

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