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High-Risk Human Papillomaviruses and Breast Cancer in Thi-Qar province/South Iraq

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Abstract:-

The breast cancer is considered one of the most common types of cancer in Iraq, and in Thi-Qar the infections are increasing annually, it constitutes one-third of all cancer types. The HPVs are highly contagious viruses. The important aspect about HPV infections is their relationship with breast cancer. The HPV has a well-known relationship with other neoplasms in other anatomic sites and may have a role in the development of breast cancer. The HPV 16 and 18, are high-risk oncogenic viruses, and they are the most common types that can be detected in malignant tumors. To confirm the role of papillomavirus in the occurrence of breast cancer and because the absence of a study to identify the HPV genotypes in Thi-Qar province, that is necessary to determine the type of vaccine in the future, therefore, this study was conducted. The conventional PCR technique was implemented to detect the presence of HPV DNA in malignant and benign breast tissue, and the real-time PCR used to determinant HPV genotypes. This study was conducted on 100 formalin-fixed paraffin-embedded divided to 80 samples with malignant breast tumors and 20 samples of benign breast tumor were considered as a control group. The results showed that 37 (37%) of samples divided as: 32 (40%) from malignant tumors, and 5 (25%) from benign tumors gave positive results for HPV with significant differences ($p<0.05$), and the genotype results showed, that the HPV16 is the dominant high-risk genotype in malignant and benign tumors by positive percentage as 35 (94.6%), followed by unknown genotype 2 (5.4%), and there is no evidence for the presence of HPV18, with significant differences. The existence of high-risk oncogenic HPV in malignant breast tissue supports its relationship with breast cancer. The HPV16 is the predominant type correlated with breast cancer in Thi-Qar province, and no evidence of the presence of HPV18. HPV16 also isolated from benign breast tumors and may have a role in the development of cancer in the future.

Key words: HPV, Breast Cancer, Genotyping, PCR, Real-time PCR.

Introduction:-

Breast cancer is the most deadly cancer in women with nearly 9000000 deaths in 2015 (WHO, 2017). It is the most common cancer all over the world (Ferlay *et al.*, 2010). Depending on the latest Iraqi Cancer Registry, breast cancer accounts for about one third of the registered female cancers in Iraq, suggested that the breast cancer is the leading cancer site among females (Aljubori, 2018). Fadhel *et al.*, (2011) indicated that, Breast cancer is the most common or frequent cancer during 2005-2009 in Thi–Qar, accounted for (16.5%) of all cases and it alone accounted for 31.2% of all cancers in females with the incidence rate of 7.2/100000 of the population. Papillomaviruses were the first DNA viruses related to malignant transformation. They are icosahedral morphology viruses with a diameter ranged from 52 to 55 nm, with double-stranded DNA containing 8000bp. They replicate and grow in cutaneous and mucosal epithelial cells and stimulate cell proliferation, forming benign tumors. Some lesions develop carcinomas (DüzgüneŞ *et al.*, 2016). The HPVs are highly contagious viruses.

HPV, to now, is one of the most common causes of sexually transmitted disease worldwide in both men and women; An estimated 6.2 million people are newly infected every year (Weinstock *et al.*, 2014). The important aspect about HPV infections is their relationship with breast cancer. The HPV has a well-known relationship with other neoplasms in other anatomic sites (anogenital, upper aerodigestive tract, and skin) (Cogliano *et al*., 2005), also HPV DNA sequences have been isolated. In addition, HPV is related with 99.7% of malignant cervical lesions, High-risk HPV types are detected in nearly 99% of cervical cancers, and approximately 70% of them are caused by HPV types 16 and 18 (Bosch, and de Sanjosé, 2003). Women with the cervix precancer had a significantly higher risk of malignant breast tumors than the general female population (Hansen *et al*., 2012). All these reasons make us expect that the virus has a role in developing breast cancer. The most common types, HPV 16 and 18, are associated with anogenital malignancy. They are the most common types that can be detected in other types of cancer (Sisk and Robertson, 2002).

To confirm the role of papillomavirus in the occurrence of breast cancer and because the absence of a study to identify the HPV genotypes in Thi-Qar province, that is necessary to determine the type of vaccine in the future, therefore, this study was conducted.

Subjects and Methods:-

Patients: A total of 100 breast formalin-fixed paraffin-embedded (FFPE) tissue of the female patients were included in this study, 80 FFPE with malignant breast cancer and 20 FFPE of benign breast tumor were considered as a control group in this study. All of the samples were collected from Al-Hussein teaching hospital and Al-Haboby teaching hospital in Thi-Qar province/Iraq. Ages of patients range from 15 to 75 years with a mean of (45.5 ± 21.0).

Extraction of Viral DNA:-

The HPV DNA was extracted from formalin-fixed paraffin-embedded tissue by using the gSYNC™ DNA Extraction Kit (Geneaid. USA) and arranged by guideline by manufacturer.

Estimation the Concentration and Purity of viral DNA:-

When the DNA was extracted, we verified its presence and purity by utilizing Nanodrop spectrophotometer (THERMO. USA), which estimated DNA concentration (ng/µl) and examination the DNA purity by reading the absorbance at (260 /280 nm).

Detection of HPV DNA by Conventional PCR:-

The iNtRONs Maxime PCR PreMix Kit (*i*-Taq) was contained all the components necessary for DNA synthesis and amplification: *i*-Taq™ DNA polymerase, dNTP mixture and reaction buffer in one tube, and was used to amplified the L1 gene by guideline by manufacturer.

Preparation of PCR master mix

PCR Master mix	Volume
DNA template	5µL
L1gene Forward primer (10pmol)	1µL
L1 gene Reverse primer (10pmol)	1µL
PCR water	13µL
Total volume	20µL

Human Papillomavirus L1 gene Primers:

Primer		Sequence (5'-3')	Product Size
HPV PCR/ L1 gene	F	GCACCACCCACAGGACAATA	574bp
	R	CAGTATCTGGAGCAGCGACA	

This primers were designed in this study by using NCBI-Genbank HPV-L1 gene accession number (KF791917.1) and primer3 plus primer design online program.

PCR condition:

PCR step	Temp.	Time	Repeat
Initial Denaturation	94°C	5min	1
Denaturation	94 °C	30sec.	30 cycles
Annealing	55 °C	30sec	
Extension	72 °C	1min	
Final extension	72 °C	5min	1
Hold	4 °C	Forever	-

PCR product analysis by Gel Electrophoresis:-

Preparation of solutions:-

- 1X TBE buffer

TBE buffer was prepared by diluting 100 ml of 10X TBE buffer by adding 900 ml distilled water and stored at 4°C (Sambrook *et.al*., 1989).

- Ethidium bromide

A stock solution was prepared by resolving 0.05 grams of ethidium bromide into 10ml of distilled water in the clean dark bottle, mix and stored at 4°C (Maniatis *et al*., 1982).

The conventional polymerase chain products were analyzed by 1% agarose gel electrophoresis, the electrophoresis was run at 150 volts for 35 minutes by using the DNA ladder that provides 12 bands at sizes: 2000bp, 1500bp, 1000bp, 900bp, 800bp, 700bp,600bp, 500bp, 400bp, 300bp,200bp and 100bp of DNA respectively. The DNA bands were seen under UV transilluminator, and the photo was taken by advanced camera.

Determent the HPV genotype16 and 18 by NEXpro™ qPCR Master Mix (Probe):-

The NEXpro™ qPCR Master Mix (Probe) was used for detection of HPV-16 and HPV-18 genotypes based amplification of L1 gene (Husnjaka *et al*., 2000) as following steps:

Real-Time PCR master mix preparation:-

PCR master mix was set up by utilizing (NEXpro™ qPCR Master Mix (Probe) and this master mix done by organization guidelines as following procedure:

PCR Master mix	Volume
DNA template	3µl
L1 Forward primer (10pmol)	1 µl
L1 Reveres primer (10pmol)	1 µl
L1 probe (10pmol)	2µl
2x qPCR master mix	10µI
PCR water	3 µl
Total volume	20µl

Human Papillomavirus 16 and 18 Primers:

Primer		Sequence (5'-3')	Product Size
HPV-16 Primers	F	GTGGTAGATACTACACGCAGTAC	114bp
	R	ATATTCCTCCCCATGTCGTAGG	
HPV-16 probe	FAM-TGTGCTGCCATATCTACTTCAGAACCT-BHQ1		
HPV-18 Primers	F	TGTGCTTCTACACAGTCTCCTG	75bp
	R	CCTCACATGTCTGCTATACTGC	
HPV-18 probe	FAM- ACCTGGGCAATATGATGCTACCAAATT-TAMRA		

These primers and probe were utilized for distinguished HPV genotypes HPV-16 and HPV-18 according to HPV-16 L1 and HPV-18 L1 gene. These primers and probes were designed in this study using NCBI-Genbank HPV-16 L1 gene accession number (AF548834.1), HPV-18 L1 gene accession number (AF548836.1) and primer3 plus primer design online program.

The Real-time PCR cycling conditions:

PCR step	Temp.	Time	Repeat
Initial Denaturation	95C	3min	1
Denaturation	95C	30sec.	40 cycles
Annealing and extension	60C	30sec	

Real-Time PCR Data analysis:-

The data 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.

Statistical analysis: -

Analysis of findings was done by a computer with the available statistical packages for social science-version 24 (SPSS). The Chi-Square (X2) were used to assess these significant differences at level $p<0.05$.

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Finding and Discussion:-

The Concentration and Purity of viral DNA:-

The viral DNA concentration ranged from (7.1 to 47 ng/µl) with a mean of 20.1 ng/µl, and its purity ranged from (1.5 to 2.6) with a mean of 1.85, as viewed in the figure (1):

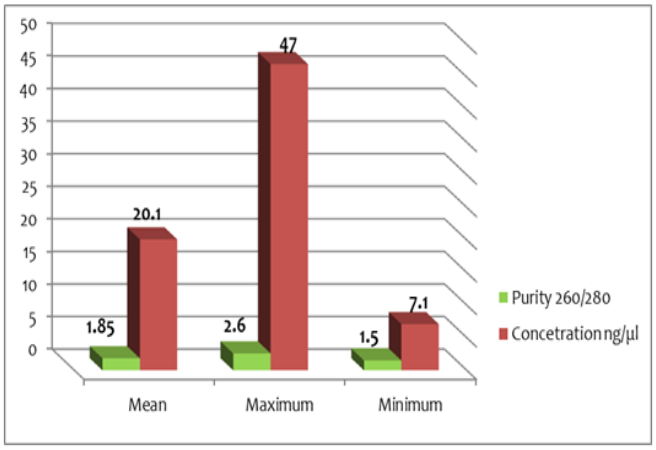

Figure (1): The Concentration and Purity of viral DNA

-Conventional PCR Detection of HPV in Breast Tumors:-

A total of 100 cases of breast tumors from female with age ranged (45.5 ± 21.0 year) gathered, comprising as: (80) malignant tumors and (20) benign tumors, were examined by PCR technique. The results showed that 37 (37%) samples divided as: 32 (40%) from malignant tumors, and 5 (25%) from benign tumors gave positive results for HPV with significant differences ($p<0.05$). (Fig.2 and 3).

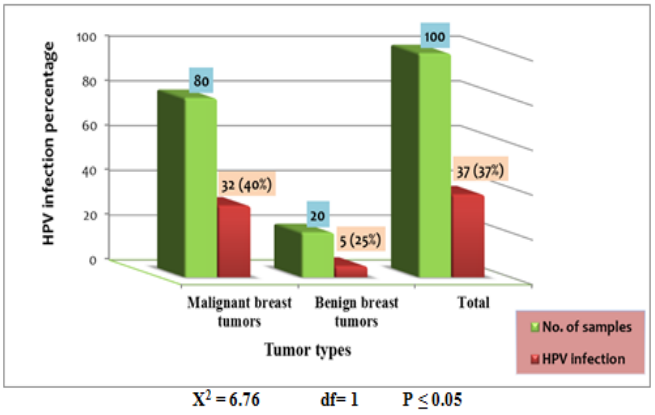

Figure (2): Human Papillomavirus Infections Percentages in Breast Tumors

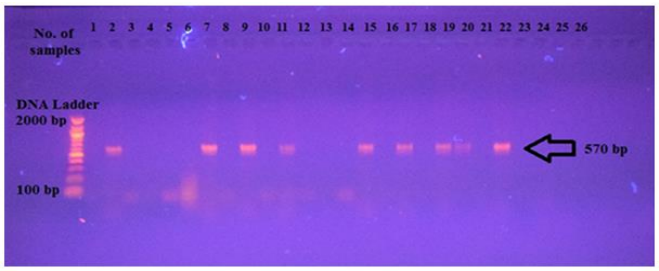

Figure (3): Bands of HPV Genotypes obtained by Conventional PCR

The breast cancer is one of the most common types of cancer in Iraq, and as Aljubori pointed out in her study in 2018, it constitutes one-third of all cancer species (Aljubori, 2018). Through our current study and previous studies on the prevalence of breast cancer in Thi-Qar province, there has been an increase in the number of infections compared to previous years ((Fadhel *et al*., 2011; Al-Mozan *et al*., 2018).

Despite the obvious role of HPV in the development of various cancers such as cervical cancer, colon cancer and head and neck cancer, its role in breast cancer remains controversial, with studies supporting and rejecting. The current findings show that 37% of breast tumor cases give positive results to HPV divided as 40% malignant tumors and 25% benign tumors with a significant difference. This finding agrees with Al. Hatemi, (2017), who revaled in his study in Thi-Qar that 32.5% of cases of breast cancer are caused by papillomavirus, But did not agree with him because no benign tumors showed any presence of the HPV. Also agrees with Ali *et al*., (2014), they estimated that 46.5% of malignant tumors and 12.5% of benign tumor positive for HPV among Iraqi patient. Isolation of the HPV16 from benign tumors is likely to be an infection at the beginning and the virus has a role in the occurrence of cancer in the late stages, or the virus has been controlled by the immune system led to a mutagenic virus that caused the loss of carcinogenic traits, we need other studies to understand the presence and role of the HPV16 in benign breast tumers.

Our results are higher than in Iran and lower than in Syria, Manzouri *et al*., (2018) 18.2% found that HPV is present in malignant breast tissue by 18% among Iranian patient in Isfahan, while Al-Moustafa *et al*., (2014) record higher percentage 61.06% among Syrian patient.

Genotyping of HPV by Real-time PCR:-

The genotype results showed, that the HPV16 is the dominant high-risk genotype in malignant and benign tumors by positive percentage as 35 (94.6%), followed by unknown genotype 2 (5.4%), and there is no evidence for the presence of HPV18 genotype, as showed in the figure (4). The statistical analysis appeared significant differences between genotypes ($p<0.05$).

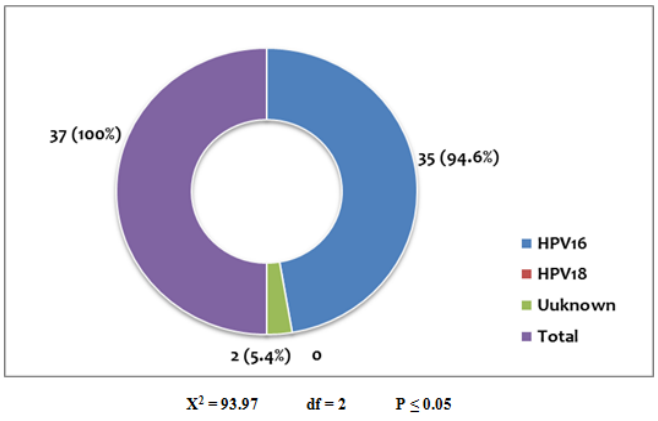

Figure (4): Genotyping of Human Papillomavirus isolated from Breast Tumors

The study of the genetic profiling of the virus helps to understand evolutionary relationships, know the source of infection and the risk factors associated with the HPV type, and also helps in determining the type of vaccine to reduce the chance of infection with the HPV.

The current study is the first of its kind in Thi-Qar province. All studies in Iraq showed several types of HPV, such as the study by Ali *et al*., (2014) they detect high risk HPV16,18, 31 and 33, and another study by Al-Mansour *et al*., (2012) they found different high-risk HPV39,52 and 59, finally the different findings by Mohamed *et al*., (2015) showed the presence of low-risk HPV6 and 11 in malignant breast tumors, All of the above studies have been used *in situ* hybridization technique to detect HPV genotypes, as opposed to our study which showed only 16 genotype along with two unknown samples and no presence of 18 genotype.

Conclusion:-

The existence of high-risk oncogenic HPV in malignant breast tissue supports its relationship with breast cancer. Human papillomavirus type 16 (HPV16) is the predominant type correlated with breast cancer in Thi-Qar province, and no evidence of the presence of HPV18. HPV16 also isolated from benign breast tumors and may have a role in the development of cancer in the future.

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