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Association of Epstein – Barr Virus with malignant transformation of breast

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Abstract--*This study was conducted to identify the Epstein –Barr virus in breast cancer the most common cancer in females for exploring possible viral association with breast malignancies. This project were included 52 formalin fixed paraffin embedded (FFPE) tissue based samples so as called as blocks as (10 benign 32 malignant and 10 healthy breast tissues as control) from females with breast tumors in the central cancer of Al-Sader Teaching Hospital additional to the private laboratory in Al-Najaf city. This study was performed on cases constituting (76.19 %) malignancy (23.81 %) benign breast lesions. The work project in methodology based on immunohistochemistry (IHC) for detecting the Epstein –Barr virus related latent membrane protein -1. These malignant samples were higher rates (37.50%) within the range (45-54) years of the patient`s ages as well as 26/32 (81.25%) positive lymph node involvement and invasive ductal carcinoma rather than invasive lobular type. In the term of viral associated breast cancer, we found a clear significance as P< 0.01 to EBV positivity in malignant breast tissues 11(34.37 %) compared to the benign at which no evidence of association had reported , even we significantly registered as P< 0.01 in the percentages of EBV with many cancer related clinicopathological characteristics such as grade 3 and T2 stage of tumor size as 6(54.55 %) for each of them additional to N3 stage of lymphatic status 4(36.36%) compared to 6 (28.57%) , 11(52.38%) and 7(33.33%) respectively for EBV negativity . In conclusion: EBV was represented a possible etiologic agent or a risk for breast cancer.*

Keywords: *Epstein- Barr virus, Immunohistochmistry Breast cancer.*

I. Introduction

The breast cancer is etiologically, multifactorial disease causes mortality and forms the most common cancer in females all over the world and in Arab countries [1]including Iraq and this is related to many factors and genes encode proteins essential for cell cycle[2] . Besides some DNA viruses included EBV and other factors shiftingbreast carcinoma towards younger ages[3]. Epsiein- Barr virus is also called human herpesvirus 4

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(HHV4),and it is(90 – 95) % prevalenthuman gammaherpes,viruscausingpersistent latent infections demonstrating a few symptomsanddetected chronically in saliva ofhealthy adults[4] even this virus can reach to epithelial tissues[5]included those of breast contribute, to epithelial, malignancies [6]. In latent stage of viral life cyclevirus exhibits genes were expressed encode latent membrane proteins (LMPs) and Epstein –Barr nuclear antigens EBNA[7]reaches epithelial breast cellsvia cell to cell contactwith infected lymphocytes**[**8**]**getting persistentlyinfected at whichintegrated within host genome inducingneoplasm development[9]. So as called oncogenic viruses as an etiologic agents that may contributetobreast malignancy deregulate host innate, immunity revealing viral and host genetic alteration. The etiology of breast Carcinoma is not yet certainly understood or clear but environment included viruses as EBV together with molecular events at the initiation stage involving genetic and epigenetic interaction contribute to breast Carcinogenesis[7]. Many of previous researches find that EBV indicated in approximately 30% of breast carcinomas in which its DNA and viral products have been detected in cancerous breast cells of a given specimens (biopsies)[10]. In term of the oncogenic mechanism, this virus predisposes breast epithelial cells to malignant transformation through cellular oncogenes HER receptors activation[11]. Latent membrane protein 1 represents a prime trans membrane onco-protein expresses in most EBV-related human cancers[12]. It comprises a short cytoplasmic terminal tail , six transmembrane domains, and long cytoplasmic C-terminus which consist of three, activating regions : denoted asCTAR1, 2 and 3as a sequence of recognition binding site deregulate many signal transduction pathways included JAK/ STAT cascade[13]leading to cellular transformation by which keep them in stem-like properties inhibiting cell differentiation , tumor ,angiogenesis , cell to cell contact and cancer invasion[14] .

In mammary tissues and breast secretion, EBV was detected[10] infecting epithelial cell and damage P31 DNA and immortalize these cells. LMP2A rises tumor initiating precursor cells promotes Mesenchymal epithelial transition (MET)[11].

EBV infected Mammary epithelial cell (MEC) undergoes mutant P 53 defective gene expression which reflects high grade BC through severe genotypic and phenotypic alterations[11]. Antiviral innate immunity may be linked to tumor development in term of EBV as a genomic modifier which acts through APOBEC3 proteins inactivating viral DNA[15] enhance its integrity to (MEC) then subsequently alters and destabilizes host genome[16].

In another hand EBV acts an epigenomic modifier implicating mechanism of histonacetyltion for post transcription modification initiate oncogenicity in EBV harbordBreast cells in asymptomatic carriers[17] even play role in lytic reactivation of virus to infect other mammary epithelial cells[17]. Other have shown epigenetic unstability in lobular breast carcinoma in which EBV – LMP 2 A activate DNMT methylome enzyme mediates hypermethylation of P53 DNA in promoter region as tumor suppressor gene correlated with metastasis[18].

II. Methodology

Sampling:

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The Breast cancer tissues in the form of (FFPE) blocks were obtained from archives of Al- Sadder hospital in Al- Najaf city and other private histopathological laboratories.Formalin fixed Paraffin embedding (FFPE) block of52 patient with breast cancer are collected (malignant ,10benign and 10 control). This case is histopathologicallyexaminedby hematoxylin and eosine staining(H & E) techniqueunder light microscope, all of these sample in Al-Najaf city. Out of these (52) samples (n=26) were invasive ductal breast carcinoma (IDBC), lobular (n=6) as malignant neoplasms. Tissues from various benign breast diseases or lesions(n=10): as fibrocystic(n=3) , fibro adenoma (n=4) additional tohyperplasia & granulomatous mastitis (n=3) as well as 10 breast healthy or normaltissueswere used as control samples. All of included cases were female patients. For TNM clinical stages there were two Cases of stage I, eighteencases of stage II,and 12 Cases of stage III and. Patient withstage I were deemed as early stage Breast cancer and those of II / III were considered as advanced stage. For pathology Grade, there were cases of Grade I, cases of Grade II, and cases of Grade III. These (FFPE) tissues were investigated with Immunohistochemistry (IHC).

Immunohistochemistry

The standard detection technique identifying EBV- LMP-1 as viral specific antigen against specific Ab. As mouse monoclonal antibody for CD21 against EBV- membrane receptor forLMP-1 involves Kit from Dako , Glostrup ,Denmark The FFPE tissue in the form of block were cut in 4µ micrometer thickness and mounted on slide, de-paraffinized and rehydrated then antigens are retrieved to be ready for immune-histo- chemical staining protocol. Primary antibody specific to EBV- LMP1 antigen is added to slide ,Horseradish peroxidase (HRP) as enzyme conjugate for antigen detection involved followed by DAB (3.3- Diamin benzidine is immune stain as a chromogen and it is oxidized by H2O2 in HRP- catalyzed reaction, forming brown pericipitate where oxidized can be visualized microscopically), the sections were rehydrated minutes to provide better refractive index then hematoxylin counter stain is applied on slides mounted with DPX examined under light microscope .

Statistical methods

SPSS version 21, Med Calc version 18.3 and Graph Pad Prism version 5 were used to analyze and graph data of this study. Comparisonsbetween two means was done by independent t-test, while ONE WAY ANOVA was used to compare more than two means and differences within groups were inspected by using multiple comparison method (L.S.D.).

Categoricalvariables were compared via Chi-squared test. Measurable data were represented as mean ±SD, whereas categorical were represented as Frequency(%).Differences between variables were setting as significant (**) at 1% ($P \leq 0.01$). No significant differences values were accompany by (ns).

III. Results

This study includes 52 FFPE block tissue based samples (10 benign 32 malignant and 10 healthy bread tissues as control) for females with breast tumors in the central cancer of Al- Sader Teaching Hospital in Al-Najaf

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city additional to the private laboratory.This study was performed on (76.19 %) malignancy (23.81 %) benign breast lesions.

Table (1) Distribution of tumor type , Histology of breast tumors andlymph node involvement in present study.

Domain	Subdomain	F(%)
Tumors	Benign	10(23.81%)
	Malignant	32(76.19%)
Histology of breast tumors	ILC	6(18.75%)
	IDC	26(81.25%)
LN involvement	+ve	26(81.25%)
	-ve	6(18.75%)

The grade of breast cancer is classified in our samples to grade I as 2/32 (6.25 %), as well differentiated carcinoma, while 17/32 (53.1 %) from samples of patients were in grade II so that described as moderately differentiated which they register higher rates among breast malignancies.Grade III is the higher grading determined among this study in which poorly differentiated breast carcinoma in the percentage of (40.6 %) and 13 patients out of 32.

Immunohistochemical analysis (LMP-1 of EBV)

The result of histopathological test of breast tissue sections showed clear differentiation between benign breast lesion and malignant tumor.The details were appeared in figure (1) A, B& C.Similar prospective future study on the same tissue samples in dependent or in relation with PR, ER, P53immunohistochemical based analysis as verification to support our findings for checking other breast cancer influencing factors.

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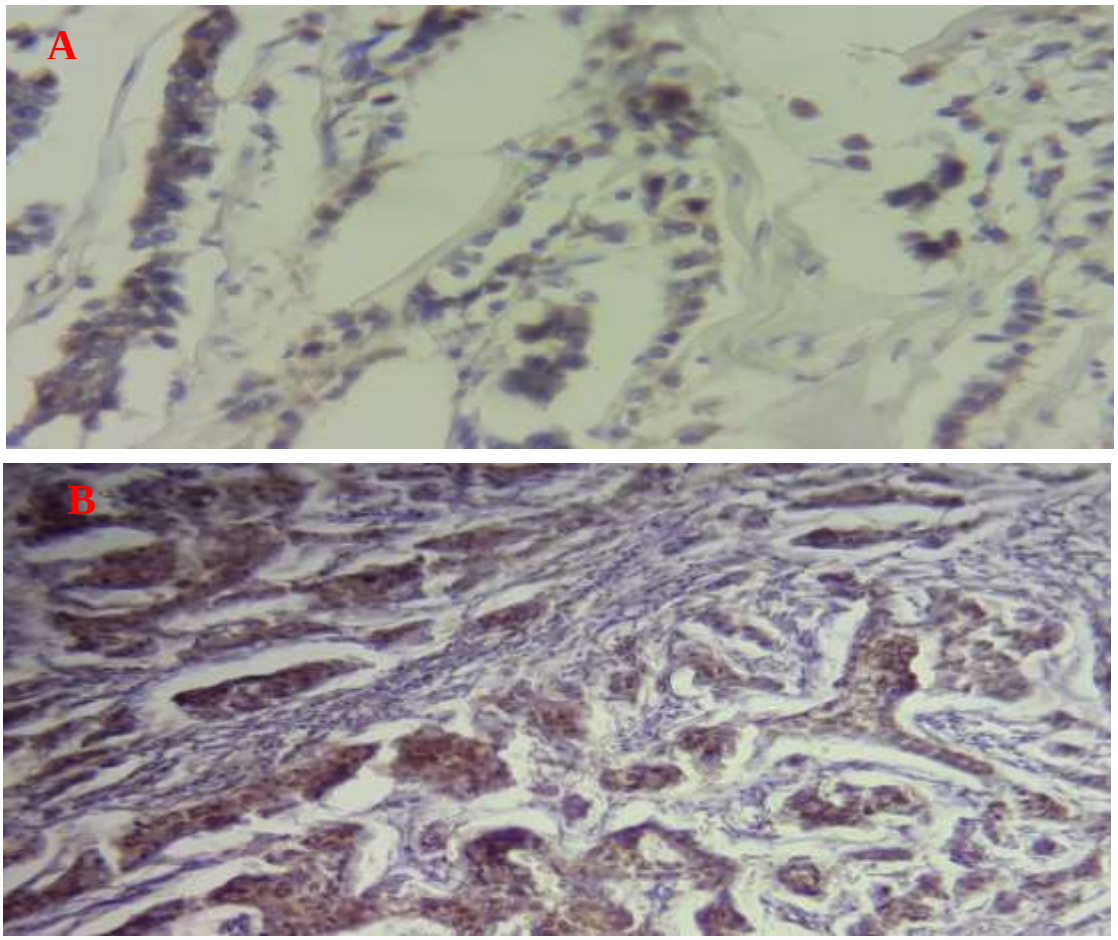

Figure (1: A &B) EBVness positiveimmunohistochemistry of malignant breast tissues

A-Positive (IHC) slide for EBV shows cytoplasm stain of invasive lobular carcinoma(ILC)

B-Positive (IHC) slide for EBV shows cytoplasm stain of invasive ductal carcinoma(IDC).

Association of Epstein –Barr viralLMP-1in the study groups of benign and malignant breast tumors .

This study showed that the EBV is detected only in malignant tumor in percentage of 11/ 32 (34.3 %) in clearly significance compared with the healthy breast control samples and even with the benign breast lesions they were also negative for this virus by means of LMP-1 antigen.

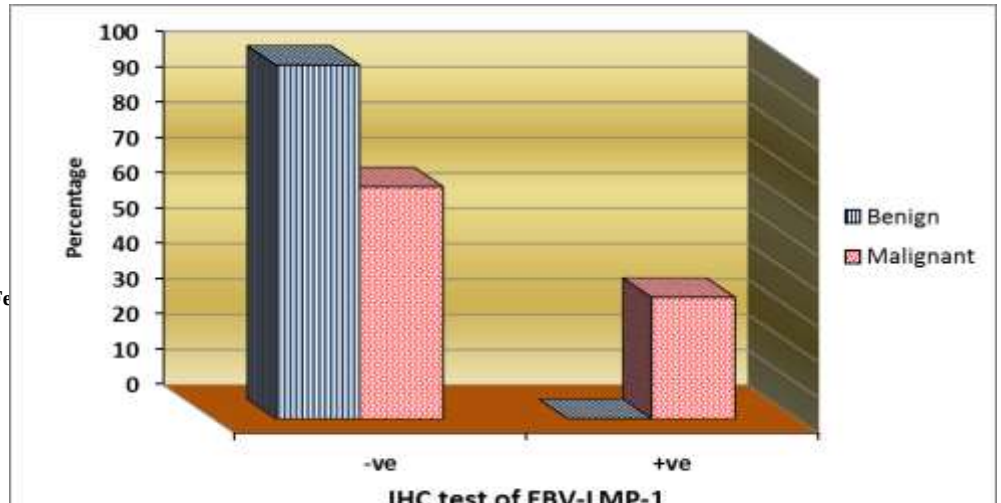

Figure (2) EBV-LMP-1 with type of breast tumor

Table (2) Percentage of EBV-LMP-1 with type ofbreast tumor

Tumor	EBV		P value
	-ve	+ve	
Benign	10(100%)	0(0%)	≤0.01**
Malignant	21(65.63%)	11(34.37%)	
Total	31(73.81%)	11(26.19%)	

**=highly significant at P≤0.01, Chi-squared (Fisher's Exact test)

AssociationofEpstein –Barrvirus EBV-LMP-1 with Grade of tumor

The grade 111 was registered higher rate 6 (54.55) than grade 11 as positive cases of EBV in comparison with EBV negative at which higher percentage 13 (61.90 %) had achieved among grade 11 with significance of P ≤ 0.01.

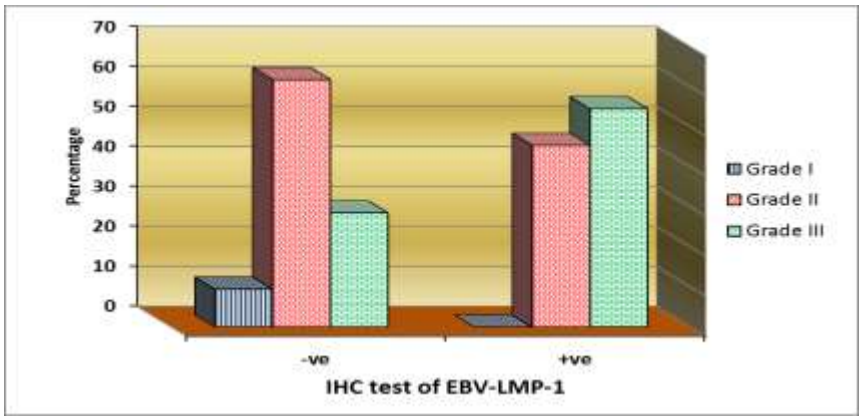

Figure (3) EBV-LMP-1 with Grade of tumor

Table (3) Percentage of EBV-LMP-1 with Grade of tumor

Grade	IHC test of EBV-LMP-1		P value
	-ve	+ve	
I	2(9.52%)	0(0%)	≤0.01**

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II	13(61.90%)	5(45.45%)	
III	6(28.57%)	6(54.555)	
Total	21	11	

Association of EBV-LMP-1 with tumor size

Among 11/ 32 cases EBV+, (7) were within T2 stages whereas the rest occurred within T3.The EBV positive breast cancer in our project as concentrated among females with T2 as 6 (54.55) and T3 stage of tumor size as 5 (45.45 %) whereas none of early and advanced stage of tumor size as (T1 and T4) registered immunehistochemical positivity to EBV in contrast with EBV negative cases which were distributed among all different stages of tumor size particularly T2 as 11 (52.38 %) at which the proportion being higher with significance association of P value ≤ 0.01.

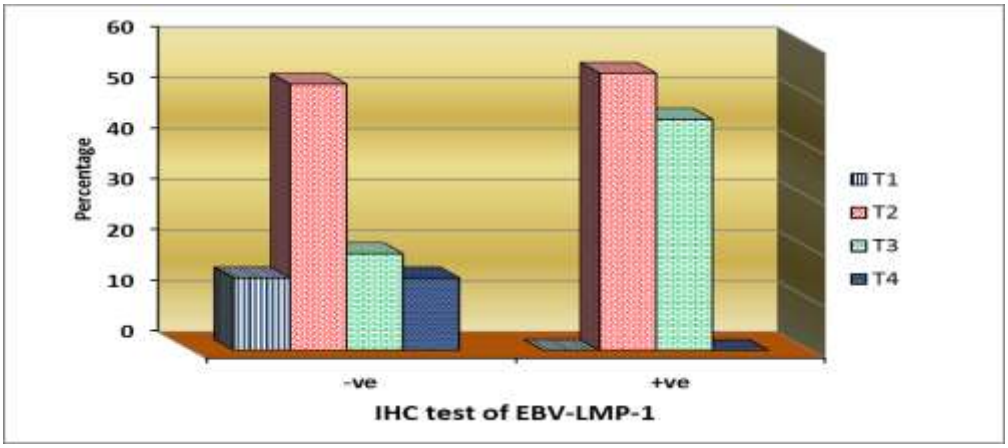


Figure (4) EBV-LMP-1 with tumor size

Table (4) Association ofEBV-LMP-1 with tumor size

Tumor size	IHC test of EBV-LMP-1		P value
	-ve	+ve	
1	3(14.29%)	0(0%)	≤0.01**
2	11(52.38%)	6(54.55%)	
3	4(19.05%)	5(45.45%)	
4	3(14.29%)	0	
Total	21	11	

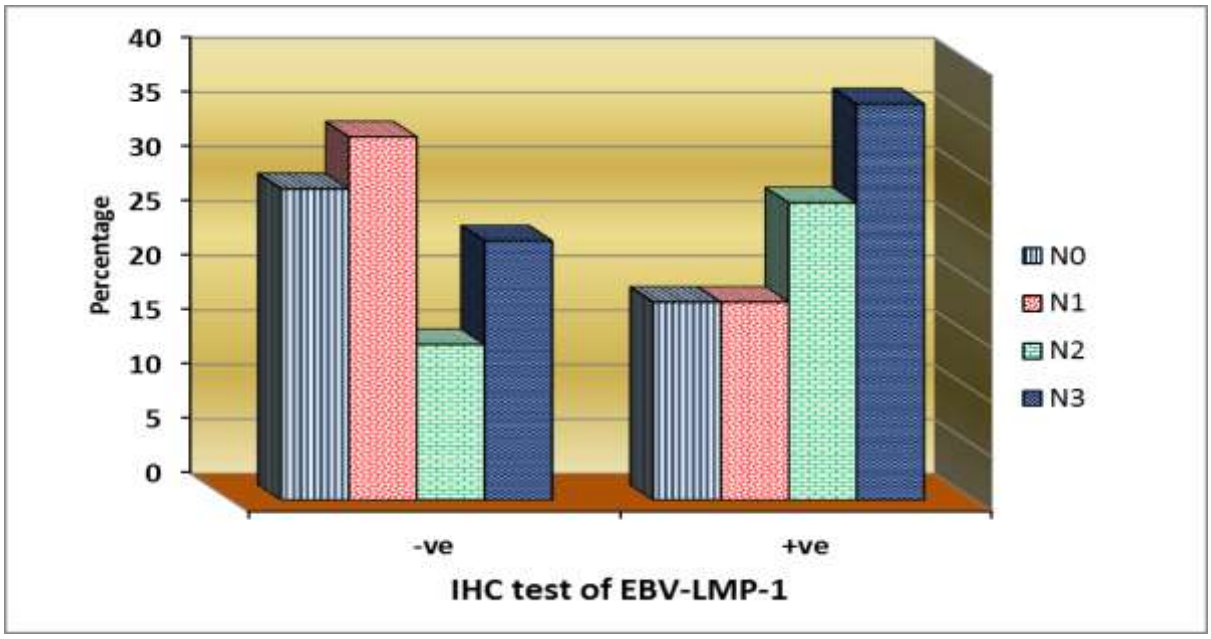
Association ofEBV-LMP-1 with Lymph node metastasis

In relation with lymphovasicalar invasion EBV had showed significant variation higher percentage of EBV positivity for breast malignant tissues was with N3 stage of lymph node metastasis ascases (36.36 %) while N1 stage in the present study had exhibited the higher rate 7 (52.36 %) among EBV negative breast cancer our tissues with P value ≤ 0.01andabout 82% of the viral positivityhad reported positive nodal status .

Table (5) PercentageofEBV-LMP-1 with Lymph node metastasis

Lymph node metastasis	IHC test of EBV-LMP-1		P value
	-ve	+ve	
N0	6(28.57.%)	2(18.18%)	≤0.01**
N1	7(33.33.%)	2(18.18%)	
N2	3(14.29 %)	3(27.27%)	
N3	5(23.80 %)	4(36.36%)	
Total	21	11	

Association ofEBV-LMP-1 with Age of malignant patients.



Our result showed the most affected range of patient's ages with breast carcinoma was mainly (45- 54) years**.** The present researchwere showedno substantial heterogenecity as p value 0.57 between Epstein Barr virus positive and negative breast malignancy . Epstein-Barr viral positive breast cancer occurred among patients with mean ages at 51 years at which statistically not significant asp value 0.57 in relation with EBV negative breast malignancies.

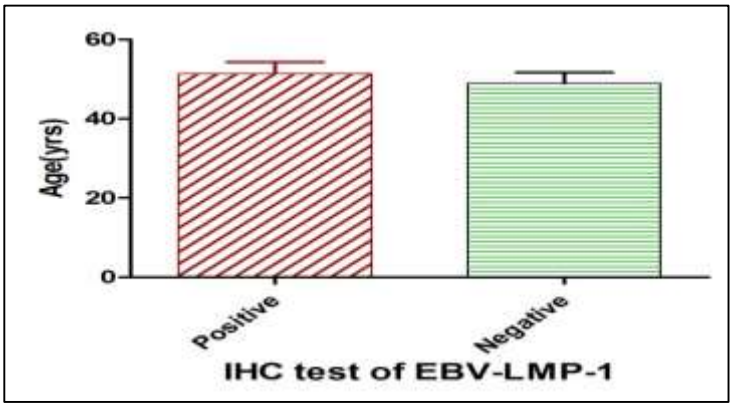


Figure (5) ofEBV-LMP-1 with Age of malignant patients

Table (6) Percentage of EBV-LMP-1 with Age of malignant patients

EBV	Age			P value
	Min	Max.	Mean(±SD)	
+ve	36	69	51.4(±9.57)	0.57
-ve	25	76	48.9(±12.60)	ns

IV. Discussion

Breast neoplasia is prevalent which has been approved in the world. EBV is potential for breast cancer related risk or etiologic agent which is firstly had proposed[19] in that 50 % of breast malignant samples were EBVness positive followed by Bonnet, et al., 1999 using molecular based detection PCR method for LMP-2 DNA in the rate of 51 % of such cases.investigation of EBV in term of LMP-1 positivity by immunohistochemistry technique was applied on the patients breast tissues in alongside possible association with many clinic pathological features. However, the implication of viral etiology of breast carcinomas has been proposed in many projects in our country for instance those of[20,21]. Despite IHC test positivity for EBV is restricted only in breast cancerous cells rather than infiltrating lymphocytes as ocured with molecular based PCR techniques but they still better and more sensitive as well as long term formalin fixed paraffinized archival breast tissues as block samples if compared with fresh or frozen tissue based ones.In this study a total 52 FFPE breast tissue blocks as case samples were collected and haematoxylin and eosin stained (haematoxylin for nuclear staining while eosin for cytoplasm) then

microscopically examined to identify them histopathologically whether benign, malignant, and normal tissues. The malignant or breast neoplasia constitute cases (76.19 %) whereas benign breast hyperplasia constitute 10 (23.81 %)the remaining (10) is normal breast tissues are peri or so as called as paratumoral tissue block in which adjacent to tumor and some of them taken as normal by core needle biopsy used as healthy control similarly to number of studies that had involved (60.7 %) malignant Syrian cases[22].

Whereas Hassb El-Naby and his collogues in their survey depend on equal proportions 50 % for both malignant and benign cases. The grade of breast cancer is classified in our samples to grade I as 2/32 (6.25 %), as well differentiated carcinoma, while 17/32 (53.1 %) from samples of patients were in grade II so that described as moderately differentiated which they register higher rates among breast malignancies.

Grade III is the higher grading determined among this study in which poorly differentiated breast carcinoma in the percentage of (40.6 %) and 13 patients out of 32. The present study comes in opposite to[23] who approved a higher rates of breast carcinoma of pathological grading I as (26.3 %) and grade III as (44.7 %) whereas lower percentage of grade II (28.9 %) had registered this variation many be changed with increase the samples number or due to earlier diagnosis of those mastectomized women.Despite the fact of the breast malignancy is common in women over the age 50 according to NCI (National cancer institute) ranging 55- 64 years during 2012- 2016 data, our result showed the most affectedrange of patient's ages was mainly (45- 54) years but this results may be different especially when several handrads of cases used. Even more in my country this data of patents are taken orally and directly from woman herself or her relative in undocumented manner so, it is subject to be inaccurate.

Furthermore, it should be taken in consideration other undetermined significant risk factors such as inherited altered or mutated genes, exposure to radiation, hormone replacement therapies, etc. As Mezher, et al collected the samples from the same hospital that we had collect they got patients women with BC with mean age of 51.6 years in compare with 54.5 years for benign tumors during 2017 in patients ranged as (26- 68) years so, this is about to be near to the present study in which the range of ages is (24- 76) in approximate with little variation.Noteworthy, the high incidence of breast cancers in younger age group may be related with environmental pollutants mainly those referred to the weapons from 1991 and to date. Therefore our results are coming in equal with many previous research’s in Iraq[24,25,3,21] in addition to the Arab countries[26,27].

This study showed that the EBV is detected only in malignant tumor in percentage of 11/ 32 (34.3 %) in clearly significance compared with the healthy breast control samples and even with the benign breast lesions they were also negative for this virus by means of LMP-1 antigen .This present findings are corresponding to previous studies in Iraq as (40 %)[20] and higher percentage (51.5 %) of EBV positivity was observed among Syrian women[22]. In contrast, some studies had suggested no evidence of association between EBV and BC for instance and globally, in thatproject had performed among Mexican women with breast malignancy[28].Neighboring to our country, only two cases out of 39 were viral positive in proportion of (5.12 %) had been indicated as no significant association or relationship between EBV and BC in Iran[29], as well as in reciprocal to[30] where they had proposed no association of EBV with BC involving FFPE in IHC and other molecular based detection

technique on 18 Iranian patients of Tahran.Globally/ it has been reported using PEPE tissue the EPV prevalence rate in lobular BC Is higher 34.78% than ductal type 28,60% and other mixed breast cancer type in the pooling of data that published from 1990 to 2010 in met analytic study of [31].Regarding the association of EBV positive with cancer grade [32] were reported as no statistical significance between them neither among Egyptian patients nor Iraqi .With histology ofBC[31]ductal type was 11\42 26.2% and 14\32 (43.8%) in Iraqi and Egyptian Patients respectively compared to those of lobular breast cancer type of histology which were higher population in the rates of 37.5% (3\5) and 50% 4\8 of EBVness positive cases in P value 0.53 and 0.40 respectively.Younger age patients ≤30 - ≤50 in Iraqi and Egypt approved higher proportion of viral positivity 29.2% (12\41) P=0.51 and 47.8% (11\23) in p=46 respectively[31].With grade EBV the cases were found among grade 1 BC women of Iraq and Egypt respectively 31.2%[31] and 46.4% respectively .

In similar study had conducted in Najaf governorate by[20] reported that strong relation between virus BC 40% LMP positive mostly inthe mean age of 59 age and in the rate of 66.6% within grade 3 involvingISH technique and PCR.Despite no connection was found between EBV and steroid hormonal receptor in the similar study[20], were reported in previous resound in that 51,6year mean age of patients with breast malignantly of 59.5 years for benign breast lesions in substantial statistical heterogeneity or significance of P. valuep≤0.05 between this two groupsof patients.

In the term of lymph vascular invasion constitute 66.67% patients exhibiting LN involvement by breast neoplasm and higher rates of EBV positivity were found among negative LN[33]with no significant. Even[20] were provide no evidence of statistical significance or correlation between EBV positive and LN metastasis .About 71.15% of EBVpositive 111 cases exhibit positive lymph node involvementin study carried and an Indian females [34] which was corresponding or coming in agreement to ours.

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