Effect of EBV-LMP1 mutations (24bp and 30bp) on the frequency of PIK3CA mutation with and without PI3K-inhibitors

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Abstract

The study was designed to elucidate the presence of Epstein –Bar virus (EBV) in invasive ductal carcinoma (IDC) by detection of latent membrane protein 1(EBV-LMP1) by conventional polymerase chain reaction (PCR). The ability of wild type EBV-LMP1 and its isolated mutations to the disrupt the signaling pathway of phosphoinositol 3 kinase catalytic subunit A (PI3KCA). One hundred fifty-six paraffin-fixed embedded blocks of breast tumor samples which were taken from women in Basrah province were included in the study; 84 samples were benign of fibroadenoma and 72 were malignant of invasive ductal carcinoma.
EBV-LMP1 and PI3KCA DNA sequencing was confirmed with polymorphisms and two EBV-LMP1 mutations; deletion of 30bp at (213-243) and 24bp at (544-568) were successfully cloned in peGFP-C1 plasmid. Immunoprecipitation assay revealed the interaction of wild type LMP1 and its mutant 24bp deletion with PI3KCA through transfection of mammalian MCF7 cell line. On the contrary, LMP1 30bp deletion was failed to interact with PI3KCA in immunoprecipitation assay and decreased the acceleration time of proliferation in wound healing assay, which may indicate the importance of this deletion in linking with PI3KCA and activation of cell proliferation. Immunoprecipitation and wound healing assay were repeated with the presence of alpelisib drug as PI3KCA inhibitor. The result confirmed that these 30bp deletion was important to interact with PI3KCA and during inhibition of PI3KCA, the EBV-LMP1-30bp was failed to bind to PI3KCA and lose the ability of LMP1 to accelerate the artificial wound healing in MCF7 cell culture. These finding stress the importance of those 30bp deletions, which may require to stop proliferation and their role in PI3KCA mutations that detected in this study in association with presence of EBV-LMP1. PI3KCA mutations of E542K or H1047R exhibited more aggressive phenotype in breast carcinoma that could assist the constitutive activation of PI3K and cell growth, especially when coupled to EBV-LMP1 oncogenes.
In conclusion, the large variability of PI3K signaling pathway and several EBV-LMP1 mutations may contribute in the emergence of therapeutic resistance, disease relapses and activation of cells proliferation.
Keywords: *m PI3KCA, EBV-LMP1 polymorphism, LMP 30bp, 24bp mutation.*

INTRODUCTION

Breast cancer is considered one of the most fatal diseases distributed worldwide. In 2020, nearly 685,000 women died from the disease around the world, accounting for 15% of all deaths in women caused by cancers 1. It is expected that the incidence of breast cancer will

reach 3.2 million new cases per years during 2050 2.

Many risk factors can induce the cell proliferation to cause cancer 3. Molecular effects of genetic alteration by mutagenic factors and exposure to ionizing radiation, other environmental factors can have an important role on the transformation of human breast cells and may induce genetic mutations and disrupt cellular pathways which may cause the tumor 4,5,

Number of studies have identified that viruses have a proved role in several types of cancers 6,7. Precursors of some viruses have been isolated from some cancers like breast cancers. EBV proteins has been detected in 30-50% of breast cancer tissue specially in more aggressive tumor in breast area. Gupta et. al. (2020)8, mentioned some evidence associating EBV positivity with higher grade tumors (0/2, 2/36, and 3/10 in grades I, II, and III by EBER1 ISH).

Some researchers explained that any mutation in any protein of the cellular pathways can cause the breast cancer as mentioned by Ortega et. al., (2020)9 when noticed in several human cancers, there were alterations or mutations in the gene encoding the catalytic subunit p110α of PI3K (PIK3CA) with more than 85% of the substitution mutations of PI3K in either exon 9 or 20. Many researchers had reported that these mutations are noticed in 18% to 40% of breast cancers 10,11.

The aim of this study is to determine the presence of oncoprotein EBV-LMP1 in breast cancer tissue, and the effects of EBV-LMP1 gene polymorphisms in cellular protein PIK3CA which required for cell proliferation and to detect protein- protein interaction between viral protein EBV-LMP1 and cellular protein of PIK3CA.

Materials & Methods:

DNA sequences: The identification DNAs were confirmed by its purification, and by sending for DNA sequencing (Base-Gene company, Netherland). Helical domain of PI3KCA which is in Exon 9 and catalytic domain in exon 20 were sequences together as a continues sequence by the company. Sequence analysis information was performed by the company through their specific website and confirmed by using online tools as (https://www.gatc-biotech.com/en/mygatc4/single-read-sequencing/mywatchbox/mysinglerun.html#watchboxlist_watchbox_div),(http://blast.ncbi.nlm.nih.gov), (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

Cloning of LMP1 and its' isolated mutations, 30bp deletion (Δ213-234) and the 24bp deletion (Δ544-568) was inserted in peGFP-C1 by (GenScript, USA) according to (Duellman &Burgess,2006)12 by using EcoRI and BamHI restriction enzymes for both DNAs and plasmid to form sticky ends in both plasmid and PCR products. Ligation T4 DNA ligase and transformation of cloned plasmid into competent bacteria E. coli strain BL21(DE3) to overexpress and then purified to get high concentration of cloned plasmid (peGFP-LMP1, peGFP-Δ213-234 and peGFP-Δ544-568 were performed by the company.

As for breast cancer, Michigan Cancer Foundation MCF-7 cells are used ubiquitously in research for breast cancer cell experiments13. Cell culture was obtained from Pasteur Institute of Iran, Tehran, Iran.

MCF7 cell line was transfected according to Duellman &Burgess, (2006)12. GFP-EBV-LMP1 and its' mutation 30 deletions (Δ213-243) and the 24 deletions (Δ544-568) were used to transfect cells separately

Alpelisib (Piqray™) drug (PiViKTO, India), was prepared by using the formulae in molarity calculator software:

https://www.graphpad.com/quickcalcs/molarityform/

Alpelisib drug, molar mass 441.47g/mol was dissolved in 226.5 ml of DMSO to prepare 1 µM/ml and stored in aliquot at −20 °C. A 0.5µM of alpelisib was added to the MCF7 cells in wound healing assay. as recommended by Krajnak et al., (2023)14. Cell transfection with GFP-EBV-LMP1 and their mutations was estimated by detection of fluorescent light emission by the inverted UV microscope to monitor the green fluorescent light of the GFP plasmid. To ensure the connection between protein EBV-LMP1 and cellular PI3KCA protein, the GFP-Trap magnetic beads were applied to participate GFP fusion protein depending on (Chromo-Tek) manufacture's protocol and as mentioned by Abdul-Sada et. al., (2017)15.

GFP-Trap lysis buffer (Tris\Cl 10 mM, pH 7.5; NaCl 150 Mm; 1x protease inhibitor cocktail) were used for lysing of interesting cell of expressing proteins.

In vitro wound healing assay also called (scratch assay) was performed in Class II cabinet, wound healing assay was performed to measure cell motility and migration of MCF7 cell line during transfection with EBV-LMP1 inserted in GFP plasmid. This assay was designed to study the effect of EBV-LMP1 on PI3KCA pathway according to Rodriguez et al., (2005)16. And to compare the effect of presence of LMP1 protein on cell proliferation with and without 5µM anti PI3K drug alpelisib (Piqray™) in MCF7 cells.

Cell suspension containing 2.5 x 105 cells/ml was created in Opti-MEMI Medium containing 10% fetal bovine serum (FBS) and seeded 2ml from this stock in each well (5 × 105/ well) in a six well plate. Plates were incubated overnight in 37ºC with 5% CO2 and let cells to grow in a monolayer for 18-24h to achieve transfection efficiencies of 85-90% confluent. The cells were confirmed to be healthy in about ⩾ 90% viable, prior to transfection.

A sterile 20µl pipette tip was used to do a scratch vertically in each well. Plate then washed gently by 500 µl phosphate buffer solution (PBS) to remove detached cells then fresh medium alone was added. Transfection were performed by using TransfeX™ transfection kit for MCF7 cell line (ATCC, USA). Each transfection reaction contained 250 µl Opti-MEMI Reduced-Serum Medium with 2.5 µg and 5.0 µl of TransfeX™ reagent. Transfection reaction then mixed and incubated for 15min at room temperature (25ºC) before added to cells in 6 well plate. Cells plates were imaged during 0h; 6h and 18h. Free cells areas were calculated by using IMAGEJ software.

MCF7 cells alone were used as a negative control, transfected with peGFP-C1 empty vector as a positive control. Cloning plasmids, peGFPC1-EBV-LMP1 and two mutations peGFPC1-EBV-Δ24LMP1 and peGFPC1-EBV-Δ30 LMP1 were prepared to transfect the MCF7 cells and imaged in 0, 6, 18h during incubation at 37ºC and 5% CO2.The experiment was conducted twice, once with presence of anti PI3K drug (Alpelisib, PiViKTO, India) to inhibit PI3K and the other without alpelisib to compare.

RESULTS

Deletion mutations appeared to be concentrated in site of 500 bp arranged from 513 to 597. The results indicated 10 of 15 (66.6%) samples (13 from malignant and 2 for benign) were mutated by deletions confined to area of 500 bp. One mutation of 30bp was shown in area of 200 bp (213-243bp) and 23bp deletion in area of 600 (677-700bp). Mutations in 500bp region extended from the end of 500bp to early 600 bp. There was a deletion repeats 12bp at (513-525 and 591-603), and

15bp at (591-606 and 597-616) positions (Tables 1, 2).

Table -1: LMP1 deletion and position in malignant M and benign B tissues.

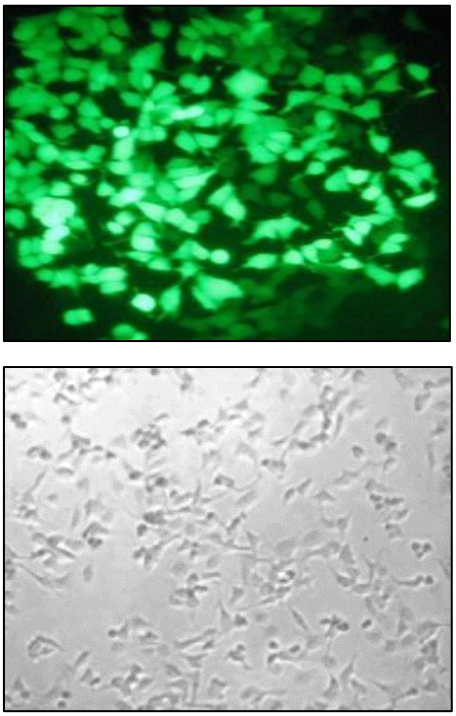
LMP1 dele/position	M		B	
	N	%	N	%
30 bp (213-243)	1	3.33		
12bp (513-525)	1	3.33		
15bp (531-546)	1	3.33		
27bp (546-573)	1	3.33		
21bp (534-555)			1	12.5
24bp (544-568)	1	3.33		
20bp (580-600)	1	3.33		
9 bp (597-602)	1	3.33		
12 bp (591-603)			1	12.5
15bp (591-606)	1	3.33		
18bp (597-615)	1	3.33		
23bp (677-700)	1	3.33		
Δ L30V	1	3.33		
Δ G187A	1	3.33		
Δ P267A	1	3.33		
No deletion/no substitution	17	56.66	6	75
TOTAL	30	100	8	100

Table- 2: local positions of PI3K mutation of breast cancer in Basrah

Types of PI3K mutation	No.	%	EBV-LMP1			
			+ve		-ve	
			No.	%	No.	%
Del. 330-350	One	6.7	0	0	One	6.7
Del. 593-602	One	6.7	One	6.7	0	0.0
Sub ΔH1047R	Five	33.2	Four	26.6	One	6.7
Sub ΔE542K	Four	26.6	Four	26.6	0	0.0
Sub ΔR88Q	One	6.7	One	6.7	0	0.0
Sub ΔR93Q	One	6.7	One	6.7	0	0.0
Sub ΔC420R	One	6.7	One	6.7	0	0.0
Sub ΔE545K	One	6.7	One	6.7	0	0.0
Total	Fifteen	100	Thirteen	86.6	Two	13.4

Monolayer of the cell culture was transfected after 24h of growing and after 18h the cell was monitored by UV converter microscope and showed that the cells were transfected well by DNA which cloned in GFP plasmid (Figure-1).

Figure -1: Cell transfected by GFP-LMP1. Left: GFP Fluorescence at 40X; Right: Phase Contrast 20X.



Cell culture of MCF7 was grown up in 6 wells Petri-dish. Cell layer was cut by tip in the middle of the growth. The cells were grown for 0, 6, 18, 24, 48h. The result showed that the cells disrupted its layer after 18h and changed to form the monolayer by 18 hours. The normal cells, which non-transfected, increased in their number and the size of the wound was decreased in about 61% during 18h. While the normal cells which incubated with alpelisib has observed to be proliferated and size of wound was 22% after 18h. Examination of cells that transfected with either GFP plasmid alone or GFP-EBV-LMP1 was revealed that the cells were proliferated quickly when infected with GFP-LMP1 as compared with transfected with GFP alone in a 100%, 54% respectively. Furthermore, proliferation of MCF7 was decreased with presence of alpelisib in both GFP and GFP-LMP1 in about 31.3% and 83% respectively (Figure -2) (Figure-3.).

The study compared the proliferation rate between wild type EBV-LMP1 and the two deletion Δ24-LMP1 and Δ30LMP1 to find whether these deletions influence the proliferation of MCF7 cell line.

Nine samples of three areas in EBV-LMP1 deletions 200-300; 500-600, and 600-700bp were sent to clone in GFP plasmid. Only two of them, Δ30bp (213-243) and 24bp (544-568) were cloned successfully by (GenScript, USA). The result of proliferation by transfection of cells with these deletions reported that the mutation Δ24 LMP1 has highly proliferated effect like the wild type LMP1 in both cultures with and without alpelisib in about 63.6% and 99.7% respectively. In contrast, the deletion Δ30 LMP1 demonstrated less effect in both cultures with and without alpelisib 33.6% and 46.8% respectively.

The MCF7 cells was transfected by GFP and GFP-EBV LMP1 to detect expression of genes and to reveal the interaction of LMP1 with PI3KCA in vitro by immunoprecipitation via GFP magnetic beads. The result demonstrated that the housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH) which used as a loading control for normalization of western blot and its bands indicates right expression. The immunoprecipitation results explicated the interaction of LMP1 and the deletion Δ24-LMP1 with PI3K which detected by their specific antibodies. LMP1 mutation which hold a 30 bp deletion (Δ30-LMP1) had lost the ability of LMP1 to interact with PI3K (Figure 2).

Immunoprecipitation assay was performed again to explain the effect of alpelisib on the expression of LMP1 and on PI3K in the cells. The result of density of bands were calculated in arbitrary densitometry units showed the expression of PI3K was decreased 72.6% when alpelisib used in cell culture. In addition, interaction of GFP-LMP1 with PI3K was indicated and the size of PI3K band which

showed binding to GFP-LMP1 was dropped in about 77.6% with presence of alpelisib in IP assay. Δ24-LMP1 showed binding with PI3KCA, and its band size also decreased in about 14.4%, whereas the interaction of Δ30-LMP1 remained missing (Figure -3.).

Figure .2: Wound healing assay, MCF7 was performed as a cell line and incision was done by edge of slide. Mock: cell with no transfection used as control. GFP, cells were transfected with GFP plasmid for positive control. GFP-EBV-LMP and two mutations GFP-EBV-Δ24LMP and GFP-EBV-Δ30LMP were transfected the cells each one individually. The squiggly lines were used to select the empty region. Transfections were used again with presence of Anti PI3K, alpelisib drug. Pictures were taken for three times 0, 6, 18 hours and areas were calculated by IMAGEJ software.

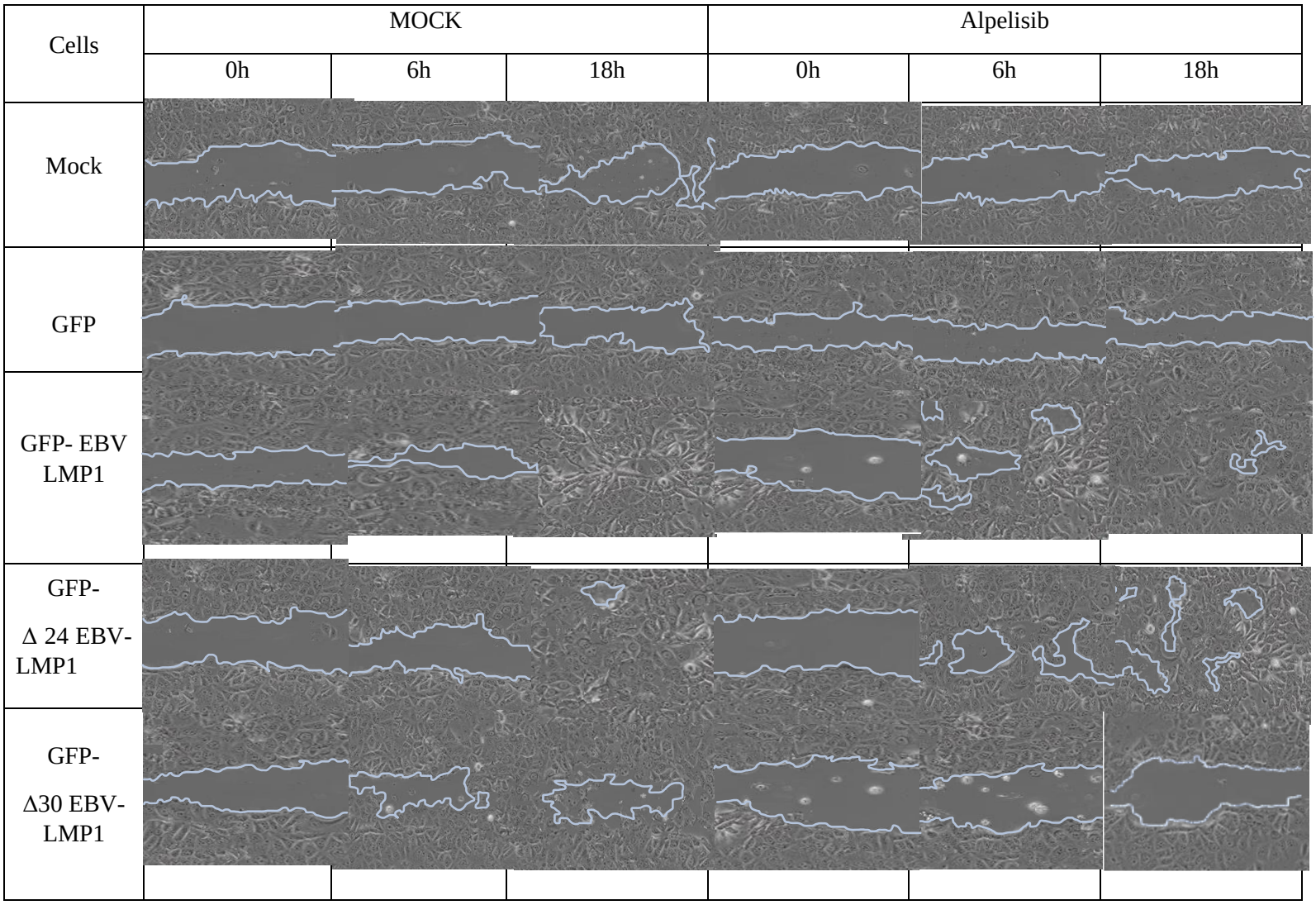
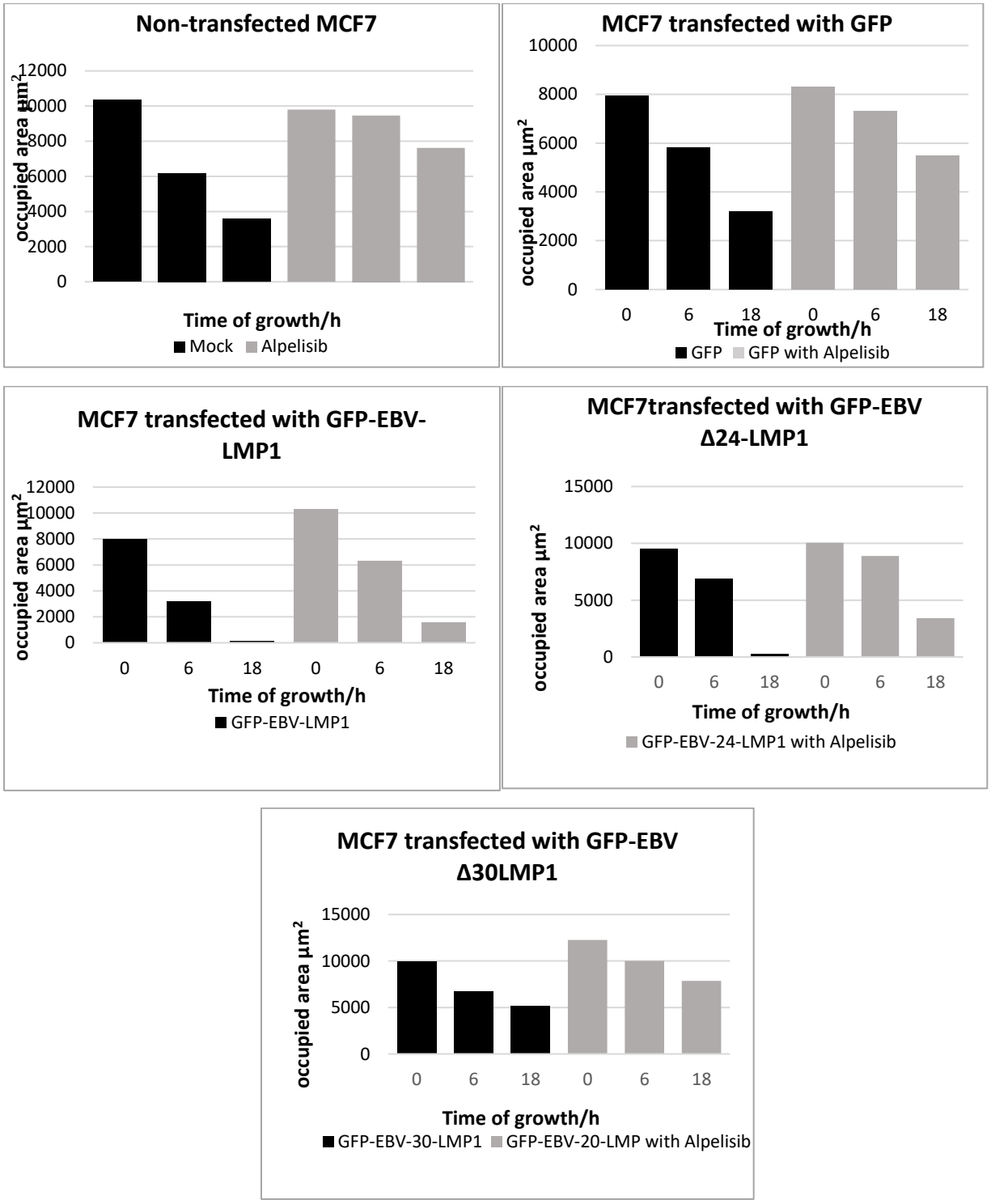


Figure .3: Bar charts to show percentage of gap closure (no cell growth) in wound healing assay in MCF7. (A) Non-transfected MCF7 negative control showed 61% clear area: (B) Transfection with GFP positive control 54.8%. (C) Transfection with GFP-EBV-LMP1 100% closure. (D) Transfection with GFP-EBVΔ24LMP1 99.7%. (E) Transfection with GFP-EBVΔ30LMP1 56%. Percentage of gap closure were calculated with and without alpelisib drug and showed the effect of the drug in growth of the cells.



Immunoprecipitation by GFP magnetic beads was performed to pull out GFP-EBVs and precipitate PI3K in cell lysate. The result referred that the wild type LMP1 and Δ24LMP1 were associated with PI3K while this interaction was lost with the deletion of Δ30LMP1 (Figures 4,5).

Figure -4: Immunoprecipitation of EBV-LMP1 and their mutations with PI3KCA. A- MCF7 cells grew and used as a mock or transfected by GFP alone or GFP-LMP and GFP-LMP deleted 24, 30 bp for 18h then lysis and proteins were detected by their specific antibody. GFP, PI3K proteins were expressed detected by gel electrophoresis, GAPDH was used to show that the gel is working well. Immunoprecipitation by GFP beads were shown. B- MCF7 transfected with presence of alpelisib (antiPI3K drug). Input and Immunoprecipitation were shown.

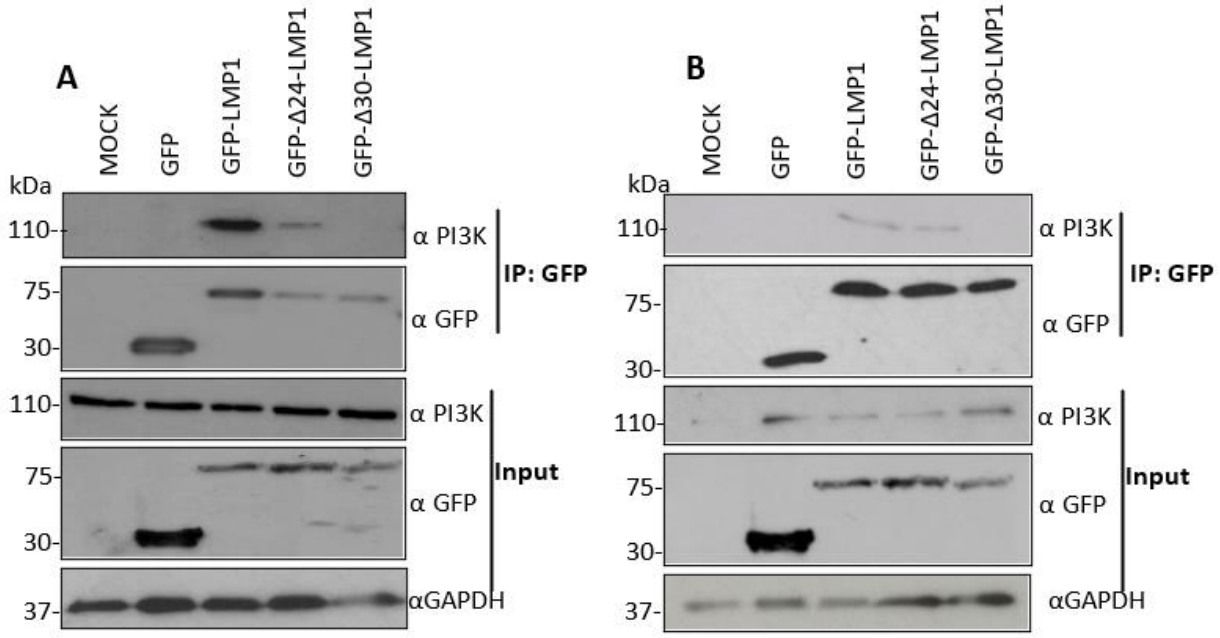
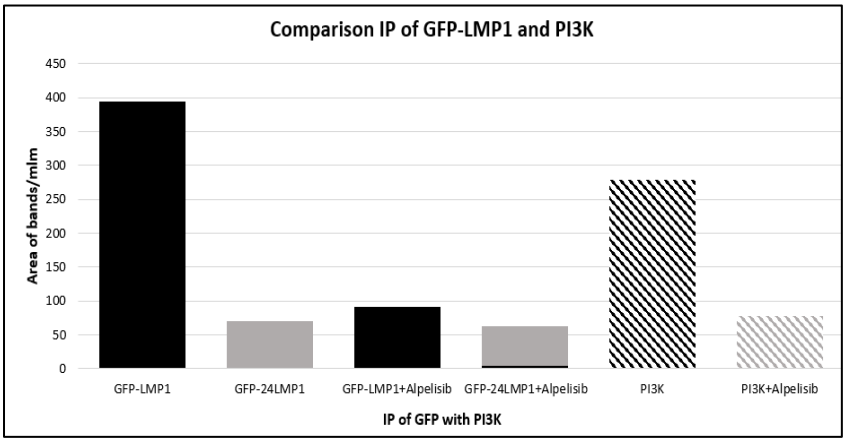


Figure -5: Density of bands were calculated in arbitrary densitometry units. GFP-LMP1, GFP-Δ24LMP, GFP- Δ30LMP1 and PI3K. Percentage of ADU was shown with and without anti PI3K drug. Immunoprecipitation density bands showed GFP-LMP1 decreased 77.6% with presence of alpelisib. GFP-24 LMP1 decreased in 14.4%, PI3K was decreased 72.6%.



DISCUSSION

In this study, the connection between the EBV oncogenic protein EBV-LMP1 and PI3KCA mutation in breast cells were shown. 8,17. The result revealed that mutation of PI3KCA increased in malignant tissues, it was observed in 93.75% which is greater than what reported in other studies when the mutations of PI3K were noticed in 20–30% of all patients with breast cancers 18,19. Researches proved that PI3K involved in several signaling pathways to induce cell growth, proliferation, regulation of apoptosis, cell survival and differentiation20,. Some studies explained that patient may take various drugs and chemotherapies that may affect the PI3KCA mutation rates22. However, the oncoprotein LMP1 and LMP2 were found in order to disrupt cell signaling pathways, one of these is PI3K/AKT pathway which is agree with the finding of this study 23, as researchers considered EBV-LMP was the activation factor for PI3K and cause mutation in it. EBV-LMP1 and 2 also plays a critical role in malignant metastasis 24,25. These findings agreed with the current results as 31% of samples were mutated in PI3K and LMP1 together in same samples.\ /PI3K mutation. The current study found that mutant PIK3CA which almost consistent with the frequencies of 33.3% (H1047R) and 26.6% (E542 K) for exon 20 and exon 9 mutations respectively, although some studies reported lower frequency (16%) for H10487R location28. This result was compatible with Bachman et. al., (2004)8 who referred to the mutant PI3K p110 subunit in breast cancer. These mutations caused by single nucleotide substitutions that form amino acid substitutions, E542K, E545K and H1047R that enhance transformation and tumorigenicity 29.

Sample that includes mutation Δ24LMP1 was associated with PI3K mutation H1047R, while Δ30LMP1 found with PI3K, E542K mutation. Studies detected mutations in exon 9 (ΔE542K), they were associated with poor prognosis, while those occurring in exon 20 (ΔH1047R) had studies better prognosis28. Four other variants of PI3K were reported in this study; ΔR93Q was mentioned and it was detected in a study with no effect in PI3K activation31. While, additional PI3K variants; ΔR88Q, C420R and E545K were detected in the results, it was recorded before to be contracted with phosphorylation of AKT, MEK1, MEK2 and protein S6 and these will increase proliferation and cell metastasis 32,33.

MCF7 cell line was performed to study migration of the cells 13,34. Cell line was transfected with GFP-EPV-LMP, Δ 24 LMP1 or Δ30LMP1 and size of the wound was measured by ImageJ software in 0, 6 and 18h. Cell monolayer was disrupted after 18h, so the study was used treatment until 18h. Results showed that the cells grow up quickly during transfection with LMP1 and the wound gap filled throughout 18h transfection. Comparing with about 61% of area was occupied in normal cells with no transfection. This result informs that the transfection with GFP-LMP1 was increased growing of the cells. The result showed that the mutation Δ24LMP1 which includes deletion between 544 to 568 bp had similar growth rate with wild type LMP. Occupation area was complete in about (99.7%) in 18h compared with non-transfected cells or those transfected with GFP alone which was about 54.8 - 61% respectively. However, the mutation Δ30LMP1 (213-243bp) showed similar effect of GFP alone and non-transfected cells (Figures 2; 3). This result informed that the 30 residues between 213-243 is required for cell transformation and its deletion led to stop proliferation of the cells like what showed in wild type LMP1.

Deletion between codon 185-211 was found to be required for TRAF binding PI3K and TRAF and transformation of primary B lymphocyte growth transformation 35. Deletion in LMP1Δ189-222 was showed to be important in activation of PI3K-AKT-mTOR. It showed that this mutation in CTAR1 of LMP1 is required to phosphorylate AKT by PI3K 36. Giehler et. al, (2022)37 studied different substitution mutation in LMP1, and they found that P204xQxT and A204xAxA were able to abolish LMP1 binding to TRAF1, 2, and 5 and confirmed their effect on the CTAR disruption in interaction with TRAF in B-cell lymphoma.

The mutation of LMP1 that lies in the 23-amino-terminal cytoplasmic tail which contains the two signaling domains carboxyl-terminal activating region 1 (CTAR1) and CTAR2 are considered to be critical in activation of PI3K pathway and NF-κB and enhances cells from G1 to S phase38.

Wound healing assay was performed by a study to detect mobility of cells during transfection with different variant of LMP1, six variants, that isolated from geographical regions called China 1, China 2, Alaskan, NC, Med+ and Med-, which identified with deletion differ from that LMP1 isolated from malignancies. These variants mutation showed slightly different in their migration cell ability, but no one had stopped the migration or inhibit it38. In the current study, wound healing assay was repeated with same transfections but with presence of anti-PI3K drug alpelisib. The results showed transformation of cells were decreased by using the drug compared with those transfected with no alpelisib. The occupied area of monolayer 61% with no alpelisib compared to 22% with presence of alpelisib. Same result was showed in transfection cells with the vector GFP and occupied area was 31.3% compared with 54.8% with no drug. This inhibition of the effect was expected according to the action of anti-PI3K drug, which inhibits subunit p110 alpha of PI3K through interaction with ATP-binding pocket domain and this act to stop converting of PIP2 to PIP3, this effect will decrease phosphorylation of AKT and S6K1 and promote cell arrest 39. Cells that transfected with LMP1 with presence of alpelisib showed little effect of alpelisib on cell growth than with non-treated cells. The area of wound was completed with no treatment during 18h, while 83% was occupied with alpelisib. This result may give a hint that LMP1 interact with PI3K and as a result it resists the drug through making a complex protein PI3K- LMP-Alpelisib, this inference was confirmed when Δ30LMP1 mutation was used to transfect the cells with presence of alpelisib, the area of cell occupied was decreased in about 33%, it means

that Δ30LMP1 has no effect on PI3K as that done by the wild type or Δ24LMP1, it may inform that the 30bp that deleted in the mutation is critical to localize LMP1 with PI3K. Localization of the oncoprotein LMP1 with the cellular protein PI3K in lipid layer of inner cell membrane was confirmed by immunofluorescence and confocal microscope, (Figure-1) and through this complex, they activate AKT pathway 40,41.

The result of immunoprecipitation with GFP magnetic beads referred that the wild type LMP1 and Δ24LMP1 were associated with PI3K while this interaction was lost with the deletion of Δ30LMP1 (Figures 4,5). This 30 bp mutation near to this area in C-terminal 185-211 was confirmed to be required for binding of CTAR1 of LMP1 with p85-PI3K and as a result enhanced cell transformation39. Immunoprecipitation between LMP1 and PI3K was studied before and confirmed that LMP1 associated with PI3K by its regulation subunit p85 42,43. The result of immunoprecipitation was confirmed binding of LMP1 with PI3K and this may be the reason why the mutation Δ30LMP1 failed to enhance cell progression like wild LMP1 and Δ24LMP1, the Δ30LMP1 may important to interact with PI3K cellular protein, specially the mutation of Δ24LMP1 was isolated from patient with grad III and mutation Δ30LMP1 was isolated from grad 1. Failure of Δ30LMP1 in its interaction with PI3K may lead the disease to remain at grad 1 and did not progress further. This finding suggests that the mutation Δ30LMP1 may stop the proliferation activity of PI3K and led to decrease cell growth rate. This hypothesis needs more confirmation to find whether these 30bp are critical for cancer progression.

It should be taken into consideration that the mutation H1047R is more aggressive phenotype isolated in breast cancer cells 44,45. Importantly, PIK3CA-H1047R recorded to enhanced fibroadenoma and adenocarcinoma 46. Study on mice that contains the mutation was revealed to promote invasive lobular carcinoma of breast and developed resistance to drugs like lapatinib, trastuzumab and pertuzumab 47,48. More experiments need to be done to confirm whether any other LMP1 mutations, which failed in cloning, be in sync with any mutation in PI3KCA and specially H1047R.

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