**Sudan University of Science and Technology**
**College of Graduate Studies**


# Molecular Detection of Epstein Barr Virus in Breast Cancer Tissue Biopsies from Sudanese Women in Khartoum State During 2015-2016

**الكشف الجزيئي لفيروس الابشتاين بار في خزعات من السودانيات المصابات بسرطان الثدي بولاية الخرطوم في الفترة من 2015-2016**

A dissertation submitted for partial fulfillment of the requirements of
M.Sc.
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# الآيــــة

قــا ل   الله  تــعا لــى:

**(وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا)**

**صدق الله العظيم**

**{الاسراء: الاية 85}**

## Dedication

*This work is dedicated*
*To*
*My parents, family and friends*

# Acknowledgement

All thank to **ALMIGHTY ALLAH** for giving me strength and courage to complete this work and made all things possible. Then thanks to my supervisor **prof.yusif fadlallah** who encouraged me to complete this work.

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## Abstract

A retrospective cross sectional molecular analysis for detection of epstein Barr virus on 51 breast cancer biopsies (paraffin embedded), previously diagnosed as breast cancer by histological examination, the samples were collected from the Military Hospital. This study was performed during the period from March to June 2017. According to their age, degree of differentiation, and type of cancer the women were divided into groups.

The specimens were treated by xylene and the paraffin removed, then the DNA was extracted followed by the detection of EBV LMP-1gene and EBV EBNA-4 gene using multiplex PCR.

Out of total 51 patients, 5(9.8%) were positive for EBV, results were compared with the positive control for EBV; while 46(90.1%) were negative.

In this study EBV was only detected in invasive ductal carcinoma but not in infiltrative lobular, ductal carcinoma *in situ*, and mucinous carcinoma. It is also concluded that EBV LMP-1gene was only detected. There was no significant association between virus detection and type of carcinoma and type of cell differentiation.

The virus was detected in age group 36-45 and 66-75 year.

**الخلاصـــــــه**


كانت هذه الدراسه رجعيه للكشف الجزيئي لفيروس الابشتاين بار لعدد ٥١عينه من خزعات سرطان الثدي (المضمنة في البرافين) والمسبق تشخيصها بسرطان الثدي عن طريق الفحص النسيجي بالمستشفى العسكري (السلاح الطبي) وتم اجراء هذه الدراسة خلال الفترة من مارس وحتى يونيو ٢٠١٧. وتبعا للعمر، درجة التفريق، ونوع السرطان في أنسجة الثدي تم تقسيم النساء الى مجموعات.

تم علاج العينات بالزيلين وإزالة شمع البرافين ، ثم تم استخراج الحمض النووي وتبعت بالكشف عن جيني الابشتاين بار LMP-1و EBNA-4عن طريق فحص تفاعل متعدد البلمرة المتسلسل.

من المجموع ٥١، أظهرت الدراسة ان ٥(٩,٨٪) اعطت نتائج ايجابيه لفيروس الابشتاين بار بالمقارنة مع المراقبة الإيجابية لفيروس الابشتاين بار ، في حين ٤٦(٩٠,٢٪) كانت سلبية.

في هذه الدراسة تم الكشف عن فيروس الابشتاين بار فقط في سرطان الأقنية الغازية ولكن ليس في مفصص ارتشاحي، سرطان الأقنية في الموقع، وسرطان المخاطيه .

وخلص أيضا إلى أن EBV LMP-1 جين هو من تم الكشف عنه فقط .لم يكن هناك ارتباط كبير بين الكشف عن الفيروس ونوع السرطان ونوع تمايزالخلية.

تم الكشف عن الفيروس في الفئة العمرية 36-45 و 66-75 سنة.

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# LIST OF ABBREVIATIONS

| ABBREVIATION | |
|---|---|
| EBV | Epstein Barr virus |
| RSV | Rous sarcoma virus |
| SV40 | Simian virus 40 |
| V-ONC | Viral oncogene |
| PTLD | Post transplantlympho proliferative deseases |
| HHV-4 | Human herpes virus 4 |
| EBNA | EBV nuclear antigen |
| LMP | Latent membrane protein |
| LCLS | Lymphoblastoid cell lines |
| TRAFS | Tumour necrosis factor receptor associated factor |
| HLA | Human leukocyte antigen |
| VCA | Viral capsid antigen |
| EA-D | Early antigen diffuse |
| EIA | Enzyme immuno assay |
| ISH | In situ hybridization |
| IHC | Immunohisto chemistry |

# CHAPTER ONE
# INTRODUCTION

## 1.1Introduction:

Cancerogenesis is a multi step process characterized by complex cellular changes resulting in uncontrolled proliferation of malignant cells often developing from a normal cell. This development is caused by the accumulation of alterations in the genes responsible for the control of cell divisions and the maintenance of genomic integrity. The development of cancer is therefore, a multifactorial mechanism initiated by multiple agents such as the environment, genetics, irradiations and also the implication of viruses (Ries *et al*., 2002).

Breast cancer is the most frequently diagnosed malignancy of women in many populations, and is the second reason of mortality in the world (Boyle and Levin, 2008).

Research into its etiology has focused primarily on reproductive and other factors affecting circulating sex hormones (Adami *et al.,* 1998; Hulka and Moorman, 2001; Henderson *et al*., 1996), and on genetic susceptibility (Rebbeck, 1999; Szabo and King, 1997; Martin and Weber, 2000). However, as identified risk factors are thought to explain only about half of all breast cancer incidences (Madigan *et al*., 1995; Mezzetti *et al*., 1998), researchers are motivated to consider other routes of disease pathogenesis. Viruses have been implicated in the development of various cancers (Serraino *et al*., 2001), but they have not been much considered for breast cancer. Identification of a mouse mammary tumor virus supports a viral etiology for breast tumors in animals, but similar germline viral sequences found in humans are not believed to play any direct role in carcinogenesis (Pogo and Holland, 1997; Wang *et al*., 2001; Labat, 1998).

Epstein Barr virus, an ubiquitous $\gamma$ herpesvirus, is a risk factor for developing Burkitt's lymphoma, nasopharyngeal carcinoma, post transplant lymph proliferative disorder, a subset of Hodgkin lymphomas, and gastric carcinomas (Crawford, 2001). In these cancers, for which epidemiologic and molecular virological data support a causal link, EBV DNA and one or more viral gene products have been detected in the majority of tumor cells (Crawford, 2001). Recently, EBV was implicated as a possible contributor to a subset of breast carcinomas (Glaser *et al*., 2004). The data bearing on this putative association of EBV with breast cancer are contradictory and therefore inconclusive. These contradictions reflect the different assays used, their different sensitivities, and different definitions of ''EBV positive'' (Perrigoue *et al*., 2005).

Approximately 70% of advanced breast cancer tumors are EBV-positive (Bonnet *et al*., 1999). 80% of EBV-positive tumors are estrogen receptor negative (Bonnet *et al*., 1999). The frequency of EBV positive tumors is also higher in breast cancer patients with three or more metastatic lymph nodes than in patients with fewer metastatic lymph nodes (Bonnet *et al*., 1999). The virus DNA was detected in 31.8% (162) of 509 primary invasive ductal breast cancer samples from geographic areas with various risks for nasopharyngeal carcinoma (Fina *et al*., 2001).

In Sudan, breast cancer is characterised by a geographically focal nature, early onset and aggressive course of the disease (Khairy *et al*., 2005). BRCA1, BRCA2 and p53 mutations are infrequent in Sudanese breast cancer patients. Epigenetic changes are suggested as alternative mechanisms to account for the minor contribution of genetic alterations in three tumour suppressor genes, BRCA1, BRCA2, and p53, in both sporadic and familial breast cancer cases in Sudan (Masri *et al*., 2002).

Viruses are likely to play a role in inducing the two categorized forms of aberrant methylation, hypomethylation, and hypermethylation (Hanahan and Weinberg,

2000; Jones and Baylin, 2002; Feinberg and Tycko, 2004) but the exact mechanism involved is yet to be understood. With or without viral involvement, the picture seems to be complex enough; the methylation profiles of tumour suppressor genes appear to vary according to tumour type, and each tumour apparently displays a distinct 'DNA hyper methy-lome'( Esteller, 2005).

## 1.2 Rationale:

Cancer is fatal disease. WHO 1991 estimated that in the next quarter of the century the number of new cancer cases globally is going to double, half of them in the developing countries.

Breast cancer is the most frequent malignancy among women in Sudan and worldwide. Despite the public health significance of the condition there are few well defined risk factors associated with the disease which could help explain its high incidence (Yahia *et al*., 2014).

Epstein Barr virus is one of oncogenic viruses and may result in cancer in different sites of body (Hodgkin's lymphoma, burkitt's lymphoma, nasopharyngeal, gastric cancer).

Several studies have investigated relationship between development between breast cancer and Epstein Barr virus eg: (Yahia *et al*., 2014), (Zekri *et al*.,2012), and (Bonnet *et al*., 1999).

The data related to the possible association of EBV with breast cancer are contradictory and therefore inconclusive. Here we searched for the occurrence of EBV in Khartoum patients with breast cancer to find association between EBV and breast cancer.

## 1.3 OBJECTIVES:

### 1.3.1General objectives:

Molecular detection of Epstein Barr virus in breast cancer biopsy samples (paraffin embedded tissue blocks) previously collected from women in Khartoum, Sudan.

### 1.3.2 Specific objectives:

1. To detect presence of EBV in breast cancer specimens using PCR technique.
2. To detect presence of EBV LMP-1 gene in tissue samples using PCR technique.
3. To investigate the association of Epstein Barr virus to various grades of breast carcinoma, degree of differentiation and their relation or association with age.

# CHAPTER TWO

# LITREATURE REVIEW

## 2.1 Viruses and human cancer:

About a fifth of all human cancers worldwide are caused by infectious agents. In 12% of cancers, seven different viruses have been causally linked to human oncogenesis: Epstein Barr virus, hepatitis B virus, human papillomavirus, human T-cell lymphotropic virus, hepatitis C virus,Kaposi's sarcoma herpesvirus, and Merkel cell polyomavirus (White *et al.,* 2014).

Several molecular mechanisms of oncogenesis that have been discovered over the decades of study of these viruses. Viruses can act at different stages in the complex multistep process of carcinogenesis. Early events include their involvement in mutagenic events associated with tumor initiation such as viral integration and insertional mutagenesis as well as viral promotion of DNA damage. Also involved in tumor progression is the dysregulation of cellular processes by viral proteins, and this has been investigated by studies in cell culture and in experimental animals and by molecular cellular approaches. Also the molecular mechanisms whereby viruses interact with the immune system and the immune evasion (White *et al*., 2014).

During the 1970s and 1980s, viral transformation of cells in culture by retroviruses such as Rous sarcoma virus (RSV) and specifically of human cells as shown by the small simian DNA tumor virus simian virus 40 (SV40) became widely used as models in cancer research laboratories. Such research received less emphasis in the 1990s, especially with the advent of research into the tumor suppressor genes (Baker *et al*., 1990).

Experimental and epidemiological data imply a causative role for viruses and they appear to be the second most important risk factor for cancer development in humans, exceeded only by tobacco usage (Zur Hausen, 1991).

The mode of virally induced tumors can be divided into two, acutely transforming or slowly transforming. In acutely transforming viruses, the viral particles carry a gene that encodes for an overactive oncogene called viral oncogene (v-onc), and the infected cell is transformed as soon as v-onc is expressed. In contrast, in slowly transforming viruses, the virus genome is inserted, especially as viral genome insertion is an obligatory part of retroviruses, near aproto-oncogene in the host genome. The viral promoter or other transcription regulation elements in turn cause overexpression of that proto-oncogene, which in turn induces uncontrolled cellular proliferation. Because viral genome insertion is not specific to proto-oncogenes and the chance of insertion near that proto-oncogene is low, slowly transforming viruses have very long tumor latency compared to acutely transforming viruses, which already carry the viral oncogene (Tsyganenko and Kovalenko, 2015).

**2.2 Epstein Barr virus and human cancers:**

The Epstein Barr virus (EBV) has been linked to the development of a variety of human malignancies, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, some T cell lymphomas, post-transplant lymphoproliferative disease (PTLD), and more recently, certain cancers of the stomach and smooth muscle. This review summarizes these associations and in particular the role of the viral latent genes in the transformation process (Baumforth *et al*., 1999).

In 1958, Denis Burkitt, an English surgeon working in Uganda, described a common cancer affecting children in regions of equatorial Africa. The climatic and geographical distribution of Burkitt's lymphoma, as it came to be known, led

Burkitt to suggest that a vector borne virus might be responsible. After this, Epstein and his coworkers identified herpes virus like particles by electron microscopy in a cell line established in culture from a Burkitt's lymphoma biopsy. Subsequently, it was shown that sera from patients with Burkitt's lymphoma had much higher antibody titres to Epstein Barr virus (EBV) antigens than did controls. As evidence accumulated for the direct involvement of EBV in Burkitt's lymphoma, seroepidemiological evidence also suggested a link between the same virus and undifferentiated nasopharyngeal carcinoma. The detection of EBV DNA in Burkitt's lymphoma and nasopharyngeal carcinoma tumor cells, and the experimental production in 1973 of lymphomas in cotton top marmosets and owl monkeys exposed to EBV, strongly suggested that this virus had oncogenic potential in both human and nonhuman primates (Baumforth *et al*., 1999).

**2.3 Epstein Barr virus and breast cancer:**

The virus was the first human virus to be directly implicated in carcinogenesis. It is a common infection affecting over 90% of the world's population (Cohen, 2000).

EBV is ubiquitous in the population; more than 90-95% of the adult world population shows serological evidence of past infection (Mandell *et al*., 1990). Receptors for the virus have been identified in epithelial cells from the oropharynx and in cells lining the uterus (Sixbey *et al*., 1987; Young *et al*., 1989), and it is postulated that the virus can directly infect such epithelial cells; *in vitro*, asubfragment of the EBV genome can immortalize epithelial cells (Griffin and Karran, 1984; Karran, 1990).

The relationship between EBV and breast cancer is controversial. Some studies have reported an EBV incidence in breast cancer tissue as high as 21–51% (Thompson and kurzrock, 2004), whereas other investigators have failed to detect EBV in any breast cancer tissue samples (Thompson and kurzrock, 2004). Possible reasons for differences between studies include: distinct EBV detection techniques,

differing EBV derived proteins or RNAs analyzed, and epidemiological variation in EBV infections or in breast cancer itself. Regardless, whether EBV is present in breast cancer and its possible etiological role in oncogenesis remain to be clarified (Thompson and kurzrock, 2004).

**2.4 The history of EBV:**

In 1958, Denis Burkitt (Burkitt, 1958) described a common cancer primarily affecting children in specific regions of Africa. Burkitt believed a virus might be responsible for the cancer, given the climatic and geographic distribution of the cases. EBV was first identified in 1964 when Anthony Epstein's group discerned virus like particles by electron microscopy in a cell line that had been established from a Burkitt's lymphoma biopsy (Epstein *et al*., 1964). Later, it was found that sera from patients with the lymphoma that Burkitt had described had much higher antibody titers to EBV than did controls without the lymphoma (Thompson and kurzrock, 2004).

The subsequent detection of EBV DNA in Burkitt's lymphoma and the experimental production of lymphomas in cotton-top marmosets and owl monkeys established EBV as the first virus clearly implicated in the development of a human tumor (Epstein *et al*., 1964).

**2.5 The EBV structure, genome and strain variability:**

It is formally designated human herpes virus 4 (HHV-4), is one of the eight known human herpes viruses. Like those of other herpesviruses, EBV virions have a double stranded, linear DNA genome surrounded by a protein capsid. A protein tegument lies between the capsid and the envelope, which is embedded with glycoproteins that are important for cell tropism, hostrange, and receptor recognition (Kieff, and Rickinson, 2007) (Fig1). Mature virions are approximately 120 to 180 nm in diameter (Epstein *et al*., 1964; Kieff *et al*., 1982). The EBV genome of approximately 100 genes (Farrell, 2005).

There are two subtypes of EBV, which differ from each other at the EBV nuclear antigen (EBNA) loci for EBNA2, -3A, -3B, and -3C (Sample *et al*., 1990). Type 1 is dominant in the Western hemisphere and Southeast Asia, whereas types 1 and 2 are equally prevalent in Africa (Rickinson and Kieff, 2007; Zimber *et al*., 1986). These isolates are distinguished by their restriction endonuclease digestion patterns and exhibit different transforming capabilities (Alfieri*et al.,* 1984; Rickinson *et al*., 1987; Takimoto *et al.,* 1989) and the ability to spontaneously enter the lytic cycle (Buck *et al*., 1999).

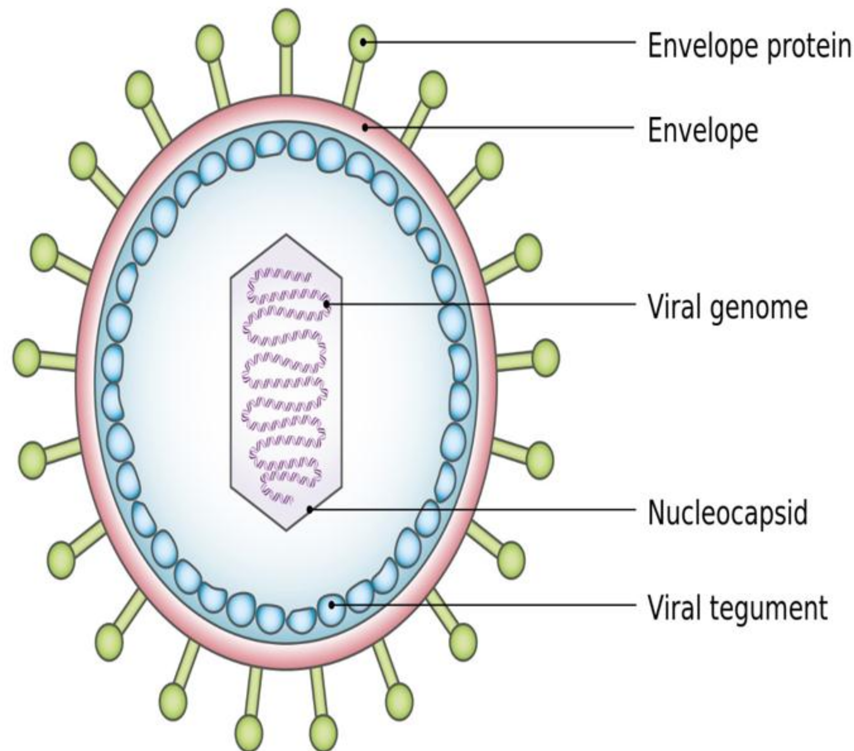



**Fig (1) EBV structure**

**2.6 Epstein Barr virus tropism:**

The term viral tropism refers to which cell types EBV infects. Epstein Barr virus (EBV) is a highly prevalent human gamma lymphocrypto virus which infects both B lymphocytes and epithelial cells (Shannon-Lowe and Rowe, 2014). In the healthy host, infection of these different cell lineages broadly reflects the different phases of the virus lifecycle. Memory B cells are the reservoir for latent EBV, in which viral gene expression is highly restricted to maintain an asymptomatic lifelong infection. In contrast, epithelial cells may be a major site of the virus lytic cycle, where infectious virus is propagated and transmitted via saliva to uninfected hosts. To achieve this dual tropism, EBV has evolved a unique set of glycoproteins in addition to a highly conserved set, which interact with cell lineage-specific receptors and switch cellular tropism during infection (Shannon-Lowe and Rowe, 2014).

The viral three part glycoprotein complexes of gHgL gp42mediate B cell membrane fusion; although the two part complexes of gHgL mediate epithelial cell membrane fusion. EBV that are made in the B cells have low numbers of gHgLgp42 complexes, because these three part complexes interact with Human-leukocyte antigen class II molecules present in B cells in the endoplasmic reticulum and are degraded. In contrast, EBV from epithelial cells are rich in the three part complexes because these cells do not normally contain HLA class II molecules. As a consequence, EBV made from B cells is more infectious to epithelial cells, and EBV made from epithelial cells is more infectious to B cells. Viruses lacking the gp42portion are able to bind to human B cells but unable to infect (Wang and Hutt-Fletcher, 1998).

## 2.7 Replication cycle:

### 2.7.1 Entery to the cell:

EBV can infect both B cells and epithelial cells (Kieff and Rickinson, 2007). The mechanisms for entering these two cells are different (Odumade *et al*., 2011).

To enter B cells, viral glycoprotein gp350binds to cellular receptor CD21 (also known as CR2) (Speck *et al*., 2000). Then viral glycoprotein gp42 interacts with cellular MHC class II molecules. This triggers fusion of the viral envelope with the cell membrane, allowing EBV to enter the B cell (Odumade *et al*., 2011). Human CD35, also known as complement receptor 1 (CR1), is an additional attachment factor for gp350/220, and can provide a route for entry of EBV into CD21negative cells, including immature B-cells. EBV infection down regulates expression of CD35 (Ogembo *et al*., 2013).

To enter epithelial cells, viral protein BMRF-2 interacts with cellular β1 integrins. Then, viral protein gH/gL interacts with cellular αvβ6/αvβ8integrins. This triggers fusion of the viral envelope with the epithelial cell membrane, allowing EBV to enter the epithelial cell (Odumade *et al*., 2011).Unlike B cell entry, epithelial cell entry is actually impeded by viral glycoprotein gp42 (Odumade *et al*., 2011).

Once EBV enters the cell, the viral capsid dissolves and the viral genome is transported to the cell nucleus (Odumade *et al*., 2011).

**2.7.2 Lytic replication:**

The lytic cycle, or productive infection, results in the production of infectious virions. EBV can undergo lytic replication in both B cells and epithelial cells. In B cells, lytic replication normally only takes place after reactivation from latency. In epithelial cells, lytic replication often directly follows viral entry (Odumade *et al*., 2011).

For lytic replication to occur, the viral genome must be linear. The latent EBV genome is circular, so it must linearize in the process of lytic reactivation. During lytic replication, viral DNA polymerases responsible for copying the viral genome. This contrasts with latency, in which host cell DNA polymerase copies the viral genome (Odumade *et al*., 2011).

Lytic gene products are produced in three consecutive stages: immediate-early, early, and late (Odumade *et al*., 2011). Immediate-early lytic gene products act as Trans activators, enhancing the expression of later lytic genes. Immediate-early lytic gene products include BZLF1 (also known as Zta, EB1, associated with its product gene ZEBRA) and BRLF1 (associated with its product gene Rta) (Odumade *et al*., 2011). Early lytic gene products have many more functions, such as replication, metabolism, and blockade of antigen processing. Early lytic gene products include BNLF2 (Odumade *et al*., 2011). Finally, late lytic gene products tend to be proteins with structural roles,like VCA, which forms the viral capsid. Other late lytic gene products, such as BCRF1, help EBV evade the immune system (Odumade *et al*., 2011).

Activation of lytic replication or reactivation from latency is key to transmission. The early products (e.g., BNLF2a) have a wide array of functions, including replication, metabolism, and blockade of antigen processing (Odumade *et al*., 2011).

Unlike lytic replication for many other viruses, EBV lytic replication does not inevitably lead to lysis of the host cell because EBV virions are produced by budding from the infected cell. Lytic proteins include gp350 and gp110 (Odumade *et al*., 2011; Lockey*et al*., 2008).

**2.7.3 Latency:**

Latency is the state of persistent viral infection without active viral production. EBV persists mostly in the memory B-cell compartment and possibly also in epithelial cells (Thorley-Lawson and Gross, 2004). Currently, it is thought that one in a million B cells carry the EBV genome in an individual after recovery from acute infection (Bornkamm and Hammerschmidt, 2001). It is generally thought that EBV genomes in latently infected B cells exist as episomes (Ambinder and Lin,

2005), although it is possible that the genomes exist as integrated DNA (Kieff *et al*., 1982; Kieff *et al.,* 1985).

In contrast to lytic replication, episomal replication during the latent phase occurs via host DNA polymerase. There is limited expression of EBNA and latent membrane protein (LMP) gene products during latency (Amon and Farrell, 2005). These include EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA leader protein (EBNA-LP), LMP1, and LMP2. Characterization of gene expression patterns in different cell lines (i.e., Burkitt's tumors and EBV immortalized lymphoblastoid cell lines [LCLs]) has determined that there are at least three different latency programs (Thorley-Lawson and Gross, 2004). By using different transcription programs, latent EBV genomes can multiply in dividing memory cells (type I), induce B cell differentiation (type II), activate naïve B cells (type III), or completely restrict all gene expression in a context specific manner (Speck, 2005; Thorley-Lawson and Gross, 2004). Only EBNA1 is expressed in the type I latency program, which is seen in Burkitt's lymphoma. CD8 T cells specific for many EBV antigens arise during the immune response to natural infection, but not for EBNA1, which contributes to evasion during latency (Blake *et al*., 1997). EBNA1 and LMP1/2A are expressed in the type II latency program, which is observed in nasopharyngeal carcinoma and Hodgkin's lymphoma. LMP1 and LMP2 are responsible for B cell activation and induction of a growth (proliferation) program (Bornkamm and Hammerschmidt, 2001). The type III latency program, in which all of the latency gene products are expressed, is often detected during acute infectious mononucleosis or in certain immune compromised individuals (Odumade *et al*., 2011).

**2.7.4 Reactivation:**

Latent EBV in B cells can be reactivated to switch to lytic replication. This is known to happen in vivo, but what triggers it is not known precisely (Odumade *et*

*al*., 2011). In vitro, latent EBV in B cells can be reactivated by stimulating the B cell receptor, so reactivation in vivo probably takes place when latently infected B cells respond to unrelated infections. *In vitro*, latent EBV in B cells can also be reactivated by treating the cells with sodium butyrate or TPA (Odumade *et al*., 2011).

**2.8 Transmission of infection:**

Infection is transmitted from host to host via saliva, and the virus passes through the oropharyngeal epithelium to the B lymphocytes. The virus enters the B cell and causes it to proliferate and spread through the B-cell compartment (Thompson and Kurzrock, 2004).

T cells respond and control B-cell proliferation. Resting EBV-infected B cells with limited antigen presentation persist at a frequency of 1 in $1\times10^{5}$-$10^{6}$cells and constitute the long term viral reservoir. Intermittently, these resting B cells will enter the lytic cycle and lyse, releasing virions back into the saliva while also infecting more host B lymphocytes (Thompson and Kurzrock, 2004).

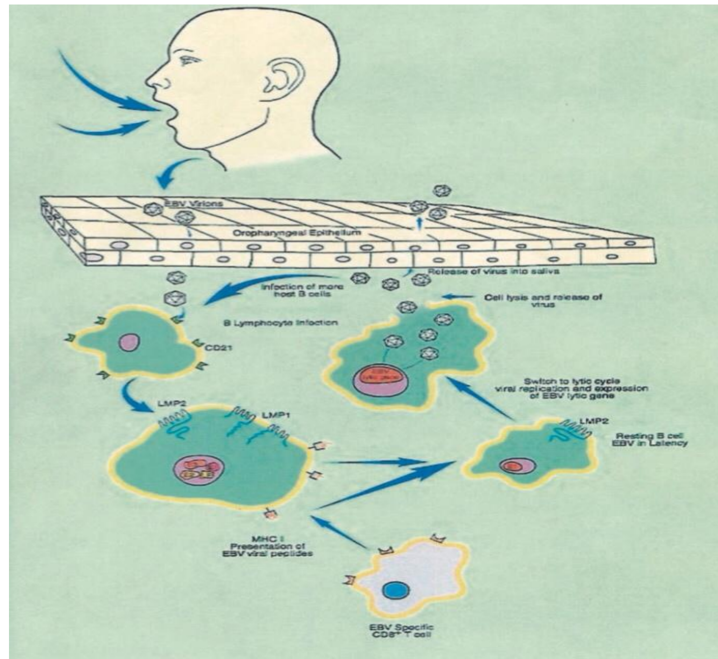



**Fig (2): Transmission of EBV (Thompson and Kurzrock, 2004).**

## 2.9 EBV latent genes and transformation:

In EBV transformed LCLs, every cell carries multiple extra chromosomal copies of the viral episome and constitutively expresses a limited set of viral gene products, the so called latent proteins, which comprise six EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and -LP) and three latent membrane proteins (LMPs 1, 2A and 2B) (Kieff and Rickinson, 2001). Transcripts from the BamHIA region of the viral genome (so called BART transcripts) are also detected in LCLs. In addition to the latent proteins, LCLs also show abundant expression of the small, non polyadenylated (and therefore non-coding) RNAs, EBER1 and EBER2; the function of these transcripts is not clear, but they are consistently expressed in all forms of latent EBV infection (Kieff and Rickinson, 2001). This pattern of latent

EBV gene expression, which appears to be activated only in B-cell infections, is referred to as 'latency III' (Young and Rickinson, 2004).

LCLs show high levels of expression of the B-cell activation markers CD23, CD30, CD39 and CD70, and of the cell-adhesion molecules lymphocyte function associated antigen 1 (LFA1; also known as CD11a/18), LFA3 (also known as CD58) and intercellular cell adhesion molecule 1 (ICAM1; also known as CD54) Kieff and Rickinson, 2001; Rowe *et al*., 1987). These markers are usually absent or expressed at low levels on resting B cells, but are transiently induced to high levels when these cells are activated into short term growth by antigenic or mitogenic stimulation, indicating that EBV induced immortalization can be elicited through the constitutive activation of the same cellular pathways that drive physiological B-cell proliferation (Young and Rickinson, 2004). The ability of EBNA2, EBNA3C and LMP1 to induce LCL-like phenotypic changes when expressed individually in human B-cell lines indicates that these viral proteins are key effectors of the immortalization process (Wang *et al*., 1990).

The role of EBV latent genes in the *in vitro* transformation of B cells has been confirmed more recently by the generation of recombinant forms of EBV that lack individual latent genes. Studies using these viruses have confirmed the absolute requirement for EBNA2 and LMP1 in the transformation process, and have highlighted a crucial role for EBNA1, EBNA-LP, EBNA3A and EBNA3C (Kieff and Rickinson, 2001).

**2.10 Latent membrane protein-1 (LMP-1):**

LMP-1 is involved in transformation by acting as a constitutively active receptor (CD40) and hence mimics the cellular growth signal that normally results from the binding of CD40 ligand (Zimber-Strobl *et al*., 1996; Gires *et al*.,1997) LMP-1 has been most directly linked to oncogenesis by virtue of its ability to recruit an array

of cellular genes. It also inhibits apoptosis by elevating levels of Bcl-2(Zimber-Strobl *et al*., 1996).

LMP-1 is an integral membrane protein with six hydrophobic membrane spanning segments and a COOH-terminal cytoplasmic tail, which contains the effector (Farrell, 1998). LMP-1 aggregates in patches on the plasma membrane that are similar to patches formed by ligand engaged growth factor receptors. Mutational analyses have demonstrated that the NH2 terminus and the transmembrane segments of LMP-1 are responsible for membrane aggregation and that this aggregation is essential for immortalization (Moorthy and Thorley-Lawson, 1993).

LMP-1 mimics CD40 by associating with the same tumor necrosis factor receptor associated factors (TRAFs) (Zimber-Strobl *et al*., 1996; Eliopoulos *et al*., 1996). The COOH-terminal domain of LMP-1 interacts TRAF-1 and TRAF-2 and with tumor necrosis factor receptor associated death domain protein (Devergne *et al.,* 1996; Thompson *et al*., 2003; Izumi and Kieff, 1997; Miller *et al*., 1997; Mosialos *et al*., 1995). TRAFs and tumor necrosis factor receptor-associated death domain interaction are mediated by separate regions of theLMP-1 COOH-terminal domain, known as transformation effector sites. Transformation effector site-1 binds TRAFs, and transformation effector site-2 binds tumor necrosis factor receptor-associated death domain. At least four signaling pathways, namely nuclear factor-B, c-Jun NH2- terminal kinase, p38 mitogen-activated protein kinase, and Janus kinase/signal transducers and activators of transcription are implicated in the function of LMP-1 (Huen *et al*., 1995; Eliopoulos and Young, 1998; Eliopoulos *et al*., 1999; Gires *et al*., 1999). These molecules affect diverse signaling cascades. Nuclear factor- B is a key transcription factor involved in regulation of cell growth and apoptosis. It also controls expression of numerous cytokines, including ones such as lymphotoxin, which is an autocrine growth factor for EBV-transformed cells (Thompson *et al*., 2003).P38/mitogen- activated protein kinase is also a

central signaling pathway and activates the ATF2 transcription factor. Meanwhile, the Janus kinase/signal transducers and activators of transcription cascade integrates with the activator protein-1 transcription factor pathway (Thompson and Kurzrock, 2004).

The activating cascades associated with LMP-1 lead to the enhanced expression of B-cell adhesion molecules (LFA1, CD54, and CD58), enhanced expression of B-cell activation markers (CD23, CD39, CD40, CD44, and HLA class II), and morphological changes such as cellular clumping (Zimber-Strobl *et al*., 1996; Izumi and Kieff, 1997; Martin *et al*., 1993; Wang *et al*., 1988; Wang *et al*., 1990). The LMP-1 interactions also cause an overexpression of proteins BCL-2 and A20, which protects the infected cell from p53-mediated apoptosis (Fries *et al*., 1996; Wang *et al*., 1996).

**2.10.1 LMP-1 structure and function:**

The Epstein–Barr virus latent membrane protein 1 (LMP1) is an integral membrane protein of 63 kDa and can be subdivided into three domains: first, an amino-terminal cytoplasmic tail (amino acids 1–23), which tethers LMP1 to the plasma membrane and orientates the protein; second, six hydrophobic transmembrane loops, which are involved in self-aggregation and oligomerization (amino acids 24–186); third, a long carboxy- terminal cytoplasmic region (amino acids 187–386), which possesses most of the signalling activity of the molecule. Two distinct functional domains referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2) have been identified on the basis of their ability to activate the nuclear factor-κB (NF-κB) transcription-factor pathway (Huen *et al*., 1995). The signalling effects of LMP1 result from the ability of tumour necrosis factor receptor (TNFR)-associated factors (TRAFs) to interact either directly with CTAR1 or indirectly by interacting with the death-domain-containing protein TRADD, which binds to CTAR2 (Eliopoulos and Young, 2001). These adaptor

proteins subsequently recruit a multiprotein catalytic complex containing the NF-κB-inducing kinase (NIK) and the IκB kinases (IKKs). This results in the activation of both the classic IκBα-dependent NF-κB pathway (involving p50–p65 heterodimers) (Eliopoulos *et al*., 2003) and the processing of p100 NF-κB2 to generate p52–p65 heterodimers134. Other kinases are recruited to LMP1 through interactions with TRAF molecules including the mitogen-activated protein kinase kinase kinases (MAPKKKs) TPL2 and TAK1, and these contribute to the activation of the NF-κB, MAPK and phosphatidylinositol 3-kinase (PI3K) pathways. ERK, extracellular signal regulated kinase; JNK, c-JUN amino-terminal kinase (Young and Rickinson, 2004). Fig (3).

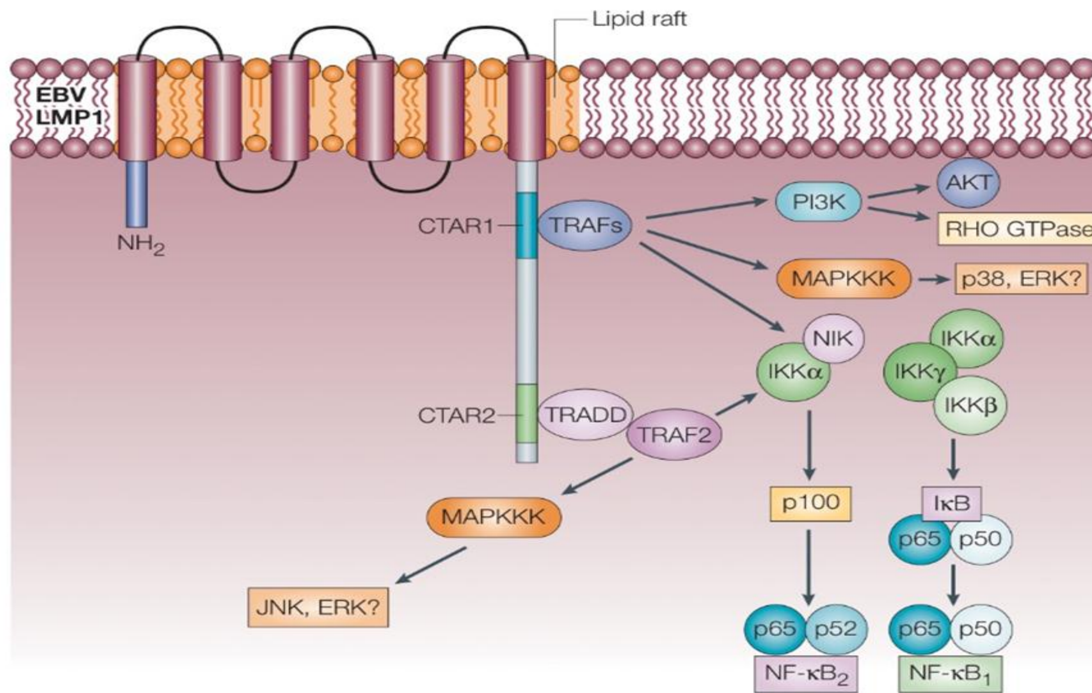



**Fig (3): structure and function of LMP-1**

**2.10.2 LMP-1Regulated Genes:**

As an oncogene, LMP1 has developed various mechanisms to interrupt normal cellular function and induce uncontrollable growth.

Many cellular genes can be up or down regulated by LMP-1. The main strategy is target groups of regulatory genes such as anti- apoptotic factors, cytokines, cell surface receptors, transcription factors, kinases, and cell cycle and adhesion molecules, all of which are essential for inhibiting abnormal growth. By altering the expression pattern of these vital genes, LMP1 confers growth advantages to cancer cells (Li and Chang, 2003).

**2.11 Immune response to EBV:**

**2.11.1 Innate immune response:**

The innate immune system is an important first line of defense against viral infections. Viruses elicit a strong type I interferon (IFN) response early after infection. This is presumed to be the case for primary EBV infection, although as alluded to above, the kinetics and quality of this response are difficult to study *invivo* because of the long incubation period. Nonetheless, EBV potently stimulates IFN production from isolated human plasmacytoid dendritic cells *in vitro* (Quan *et al*., 2010). Viral DNA and protein are recognized by pattern recognition receptors such as Toll-like receptors (TLRs), which can trigger an IFN response, facilitate the activation of natural killer cells, and act in multiple ways to prime the adaptive immune response. Our laboratory recently defined the transcriptional profile of human blood during primary EBV infection, and both type I and type II interferon regulated genes were strongly up regulated (Odumade *et al.,* 2011).

There is evidence for the involvement of multiple TLRs in activating the innate response to EBV, including TLR2 (Ariza *et al*., 2009), TLR3 (Iwakiri *et al*., 2009), TLR7, and TLR9 (Quan *et al*., 2010). Interestingly, the virus may also have mechanisms for controlling TLR signaling (Martin *et al*., 2007).

The inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-1β are increased in tonsillar tissue from patients with infectious

mononucleosis (Foss *et al*., 1994). Many studies have detected inflammatory cytokines in the sera of individuals with infectious mononucleosis. Prominent among these is IFN- γ. IFN-γ is produced by activated T cells and NK cells. Not only is IFN-γ itself elevated, but the catabolic product neopterin, which is produced by monocytes that are stimulated with IFN-γ is also elevated. IFN-γ is thought to be important for control of EBV infection and reactivation, based on studies of a related gammaherpes virus infection in mice (Ebrahimi *et al*., 2001; Lee *et al.,* 2009; Weck *et al*., 1997). However, high levels of IFN-γ likely contribute to the symptoms experienced during infectious mononucleosis, as this cytokine is known to cause headache, fatigue, and fever (Schiller *et al.,* 1990). Interestingly, type I interferon (including IFN-α) is not detected consistently in the sera of infectious mononucleosis patients (Linde *et al*., 1992; Prabhu *et al.,* 1996; Williams *et al*., 2005). This may reflect both the fact that IFN-α can be difficult to detect and the fact that it is more likely to be produced early in the response to viral infection, before the onset of infectious mononucleosis symptoms and presentation in the clinic (Odumade *et al.,* 2011).

The inflammatory cytokines TNF-α and IL-6 are also elevated during acute infectious mononucleosis. Finally, serum IL-2 is elevated during infectious mononucleosis, consistent with the dramatic expansion of CD8 T cells (Odumade *et al.,* 2011).

The immunosuppressive cytokines IL-10 and transforming growth factor beta (TGF-β) are also detected in the sera of infectious mononucleosis patients. Interestingly, the EBV late gene BCRF1 acts as an IL-10 homologue and shares 84% of its amino acid sequence with human IL-10 (Moore *et al*., 1990). During acute infectious mononucleosis, both viral and host forms of IL-10 are detected in sera (Taga *et al*., 1995). Host IL-10 is produced by monocytes and lymphocytes, functions to suppress T-cell proliferation and cytokine production, and can inhibit

IFN-γ production from T cells (Mosser and Zhang, 2008). Thus, it might be predicted that IL-10 counters the pathogenic effects of IFN-γ during infectious mononucleosis. Consistent with this, the highest levels of IL-10 were observed in patients with shorter durations of symptoms (Wingate *et al*., 2009). Furthermore, high levels of IL-10 are observed in PTLD patients and are reduced as PTLD resolves with an effective antiviral response. Thus, overall, it would appear that IL-10 and IFN-γ play key roles in the balance of immune protection and symptoms during infectious mononucleosis (Odumade *et al*., 2011).

Natural killer cells are another important component of the immune response and are thought to play a key role in regulating chronic viral infections (Lanier, 2008). In fact, human NK cell deficiencies are associated with increased susceptibility to several viral (and bacterial) infections, including EBV infection (Orange, 2002). NK cell numbers increase during infectious mononucleosis (Tomkinson *et al*., 1987, Williams *et al*., 2005; Zhang *et al*., 2007). Interestingly, their numbers are associated inversely with disease severity (Williams *et al*., 2005), suggesting that NK cells could play a role in limiting viral replication (Odumade *et al*., 2011).

### 2.11.2 Adaptive immune response:

Both humoral and cellular immune responses are generated. The humoral or antibody response is critical in diagnosing infectious mononucleosis, and the cellular response (particularly the CD8 T-cell response) is critical for controlling viral replication but may also contribute to the severe symptoms of infectious mononucleosis (Odumade *et al*., 2011).

The first humoral response detected is an IgM class antibody directed against the viral capsid antigen (anti-VCA IgM). The development of anti-VCA IgG antibodies peak during the first 2 to 4 months and then persist for life (Odumade *et al*., 2011).

IgG antibodies to the latent antigen EBNA1 do not develop in most individuals for about 3 months, but once they appear, they persist for life (Rea *et al*., 2002). Antibodies to the early antigen diffuse (EA-D) are also elicited during acute infection but they are not diagnostic of a specific phase of EBV infection and hence are not generally useful (Hess, 2004).

Anti-gp350 antibodies may be detected after natural exposure to EBV or in response to gp350 subunit vaccines (Gu *et al*., 1995; Moutschen *et al*., 2007; Rees *et al*., 2009; Sokal *et al*., 2007).

Furthermore, it has been suggested by Turk et al. that gp350 antibodies enhance epithelial cell infection (Turk *et al*., 2006). This could imply another form of immune evasion, this time from neutralizing antibodies of the humoral arm that allow EBV to be maintained in an alternate reservoir (tonsillar epithelium) when its initial reservoir (B cells) is being depleted by an active immune response (T cells) (Odumade *et al*., 2011).

Both CD4 and CD8 T cells make a robust response to EBV antigens, and over 50 HLA class I and class II epitopes have been identified for this virus (Hislop*et al*., 2007). Early in infection, CD8 T cells specific for lytic antigens tend to dominate the response, while CD4 and CD8 T cells specific for latent antigens do not show such a large burst but persist for life (Precopio *et al*., 2003). The massive lymphocytosis in the blood that characterizes infectious mononucleosis is thought to consist largely of CD8 T cells specific for EBV lytic antigens (Hislop *et al*., 2007), although possible activation of bystander cells (non EBV-specific T cells) has not been ruled out by rigorous means. This large adaptive immune response is thought to be responsible for the major symptoms of infectious mononucleosis, as disease severity correlated more closely with lymphocytosis than with viral load (Silins *et al*., 2001). Interestingly, EBV-specific CD8 T cells were found to be under represented in tonsils compared to blood early during infection. This

resolved later, suggesting that efficient control of EBV infection requires tonsillar homing of CD8 T cells (Hislop *et al*., 2010).

Ultimately, CD8 T cells are critical for control of EBV, as evidenced by the occurrence of EBV lymphoproliferation and lymphomagenesis in immunosuppressed patients (Okano, 2001) and the efficacy of EBV-specific CD8 T-cell therapy in controlling PTLD (Heslop *et al*., 2004).

### 2.12 Diagnosis:

Primary EBV infection can be diagnosed with certainty only by utilizing the appropriate laboratory tests. Patients who are mildly ill are unlikely to be identified because either they do not seek medical attention or EBV infection is not considered in the differential diagnosis (Odumade *et al*., 2011).

Patients with a typical infectious mononucleosis syndrome are still a diagnostic challenge because their signs and symptoms are not very sensitive or specific for EBV infection. For example, a recent report found that the classic triad of fever, sore throat, and lymphadenopathy had a sensitivity of 68.2% and a specificity of 41.9% for EBV infection (Grotto *et al.,* 2003).

Several signs and symptoms point to an EBV etiology. These include a very sore throat that appears inflamed and swollen and sometimes has a membranous exudate, symmetrical posterior cervical and postauricular lymphadenopathy, and eyelid edema, often accompanied by facial puffiness. Clinical findings that militate against EBV infection are rhinorrhea, cough, and rash, unless the patient is taking lactam antibiotics, in which case the rash is due to transient hypersensitivity to penicillin derivatives induced by EBV (Balfour *et al*., 1972; Patel, 1967).

#### 2.12.1 EBV-specific antibody tests:

Indirect immunofluorescence assays or EIAs are the common platforms for the detection of EBV-specific antibodies, the profile of EIA antibodies present distinguishes acute primary, convalescent, and past infections. Acute primary EBV infection is characterized by IgM antibodies to the early antigen VCA in the absence of IgG antibodies to the latent antigen EBNA1. VCA IgG antibodies may be present in acute infection, but in smaller quantities than VCA IgM antibodies. During convalescence (from the third week to the third month after onset of illness), VCA IgM antibodies dwindle, while VCA IgG antibodies rise and persist for life. Between the third and sixth months, VCA IgM antibodies disappear, whereas EBNA1 IgG antibodies become detectable and persist for life. All 3 antibodies may be present in late primary infection or subclinical reactivation, which can be distinguished from each other by performing an IgG avidity assay (Nystad and Myrmel, 2007). An evidence based correlation of serologic patterns with stages of EBV infection was recently published (Klutts *et al*., 2009). This analysis included heterophile antibody and EA-D IgG antibody in addition to VCA IgM and IgG and EBNA1 IgG (Odumade *et al*., 2011).

**2.12.2 Viral detection and quantitation:**

PCR is the technique of choice for detecting and quantifying EBV in body fluids and can also be used to quantify the virus in tissue samples (Gulley and Tang, 2010). While there are options in terms of platforms, volumes, probes, and targets, a multicenter comparison of different real-time PCR assays suggested that if samples are tested at one center on the same platform, real-time PCR is a precise technique for measuring viral load (Hayden *et al*., 2008). However, substantial quantitative differences were found when samples were tested in different laboratories (Odumade *et al*., 2011).

**2.12.3 Other tests:**

EBV can be identified in tissue samples:

#### 2.12.3.1 Southern Blot Hybridization:

Is less sensitive than PCR for detecting viral DNA but permits semi quantification of viral load (Glaser *et al*., 2004).

#### 2.12.3.2 *In Situ* Hybridization(ISH):

Permits visualization of the virus or its transcripts and thus differentiates EBV in tumor cells from EBV in surrounding lymphocytes.

When targeting EBERs, ISH is a highly sensitive and specific assay that is considered the gold standard for detecting latent EBV (Gulley, 2001; Gulley *et al*., 2002).

#### 2.12.3.3 Immunohistochemistry (IHC):

Targets and localizes viral proteins, thus distinguishing EBV in tumor cells from EBV in lymphocytes.IHC targeting LMP1 is a widely employed assay that is sensitive but limited by the fact that LMP1 is absent in some otherwise EBV-related tumors (Glaser *et al*., 2004).

IHC targeting EBNA1 is a less well-established assay and, although sensitive, not necessarily specific for EBV due to cross-reactivity with cellular proteins (Brink *et al*., 2000; Chu *et al*., 2001).

## 2.13 Treatment:

The optimal management of EBV associated tumors remains unsatisfactory (Thompson and Kurzrock, 2004).

### 2.13.1 Antivirals:

There are several antiviral compounds, They include ganciclovir, famcyclovir, acyclovir, valaciclovir (a pro-drug of acyclovir), foscarnet, and cidofovir (Thompson and Kurzrock, 2004).

### 2.13.2 Gene therapies:

Use of gene therapy constructs to express either cytotoxic or inhibitory proteins selectively in tumour cells (Young and Rickinson, 2004).

### 2.13.3 Chemotherapeutic agents:

Demethylating agents: such as 5-azacytidine are able to depress lytic, as well as potentially immunogenic (Young and Rickinson, 2004).

Hydroxyurea: is able to inducethe loss of EBV episomes in in vitro models (Young and Rickinson, 2004).

### 2.13.4 Immunotherapy:

Adoptive immunotherapy using EBV-specific CTLs, although time consuming and work intensive, may overcome this disadvantage (Gottschalk *et al*., 2002).

### 2.13.5 Monoclonal Antibodies:

The anti-CD20 monoclonal antibody designated Rituximab has enjoyed significant success in the treatment of a variety of CD20-expressing lymphomas. It is also an effective agent in the management of EBV-related lymphoproliferative disorders (Thompson and Kurzrock, 2004).

## 2.14 Prevention:

### 2.14.1 Minimizing Exposure to EBV:

(Odumade *et al*., 2011).

### 2.14.2 Antiviral Prophylaxis:

Antiviral drugs (acyclovir, valacyclovir, ganciclovir, and valganciclovir) are routinely given to patients for 3 to 6 months after transplantation to prevent or suppress herpesvirus infections (Odumade *et al*., 2011). Antiherpesvirus drugs clearly reduce the incidence and severity of posttransplant CMV disease (Hodson *et al*., 2005; Kalil *et al*., 2005) but their role in the management of posttransplant EBV disease has not been established (Humar *et al.,* 2006; Smith *et al*., 2007).

### 2.15 Vaccination:

#### 2.15.1 gp350 subunit vaccine:

The EBV envelope glycoprotein, gp350 (formerly known as gp340 or EBV induced cell membrane antigen) has been considered an attractive immunogen ever since it was shown to neutralize the virus (Pearson *et al*., 1970).

#### 2.15.2 CD8 T-cell peptide epitope vaccine:

Another strategy to control expansion of EBV infected B cells and prevent infectious mononucleosis is to generate CD8 T-cell immunity to EBNAs (Khanna *et al*., 1992).

# CHAPTER THREE

# MATERIALS AND METHODS

### 3.1 Study design:

Retrospective descriptive cross sectional study.

### 3.2 Study area:

The study was done at Sudan University for Science and Technology. Sections of paraffin embedded tissue of breast cancer histopathologically confirmed were selected from department of histopathology in Military Hospital during the period from march to june 2017.

### 3.3 Study population:

Patients with breast carcinoma attending to the Military Hospital.

### 3.4 Sampling:

Sample frames include all women with breast cancer in the above mentioned Hospital were selected randomly.

### 3.5 Sample size:

Fifty one samples were included.

### 3.6 Sample:

Treated breast biopsies sections (Paraffin embedded) were collected.

### 3.7 Inclusion criteria:

Breast biopsies which were previously diagnosed as positive for breast cancer.

### 3.8 Exclusion criteria:

Breast biopsies which were previously diagnosed as negative for breast cancer.

### 3.9 study variables:

#### 3.9.1 Dependant variables:

Presence of EBV.

#### 3.9.2 Independent variables:

Age.

Degree of differentiation.

Type of breast carcinoma.

### 3.10Ethical consideration:

Ethical clearance was obtained from the research committee of college of post graduate studies of the Sudan University for Science and Technology.

### 3.11 Data collection:

Data collected by referring to the patients files.

### 3.12 Experimental work:

#### 3.12.1 Collection of specimen:

From each breast cancer patient's paraffin block, small sections of 10µm were collected into a screw capped Eppendorf tube. To avoid cross contamination, each block was cut with new gloves and new disposable microtome blade.

#### 3.12.2 De-paraffinization and re-hydration of sections:

One ml of xylene was added to a screw capped Eppendorf tube containing sections of breast cancer embedded in paraffin then were vortexed and left for 10 min. Then sections were then centrifuged at 14,000 rpm for 10 min and the supernatant was aspirated, this step was repeated twice. Then one ml of different ethanol concentrations (100%, 80%, 50% Appendix III)were added to each sample respectively and centrifuged at 14,000 rpm for 10 min and the supernatant was aspirated. Then one ml of sterile $dH_2O$ was added and were incubated at 4°C overnight.

### 3.12.3 DNA Extraction:

#### 3.12.3.1 Digestion of protein:

The pellet obtained according to the previous steps was treated with 700µl nucleic lysis buffer (Appendix III) and 50µl of proteinase K, then incubated at 65°C for 24 hours.

An additional 50µl proteinase K was added next day and was incubated for an additional 24 hours at 65°C.

#### 3.12.3.2 Precipitation and isolation of DNA:

Ten microliter of RNAse was added followed by addition of 250µl of 6 M NaCl (Appendix III) and was let at room temp for 10 min. Then was centrifuged at 14,000 for 10 min, and the supernatant was transferred to a clean tube. Then 1ml of ice-cold (-20°C) 100% ethanol was added, the sample was mixed carefully and placed at -20°C for 20 min. Then was pelleted at 14,000 rpm for 10 min and the supernatant was carefully discarded. The pellet was washed with 1.5 ml of 70% ethanol and centrifuged at 14,000 rpm for 10 min then the supernatant was

carefully discarded and the pellet was air dried on the bench top for 10 to 15 min. Finally 50 µl of TE buffer (Appendix III) was added and was stored at -20$^{O}$C for further usage.

**3.12.4 Gel electrophoresis of extracted DNA:**

The purity of the extracted DNA was determined by running the DNA samples on 1.5% agarose gel (Sambrook *et al*., 1989).

**3.12.5 Polymerase chain reaction – PCR:**

All DNA samples were screened by EBV LMP-1primer (diagnostic band 161bp) and EBV EBNA-4 primer (diagnostic band 230bp) with the presence of control positive for EBV. The primers were synthesized by (Macrogen, Korea).

PCR was done by Multiplex PCR, amplification was done using TECHNE® Ltd peltier thermal cycler (Germany).

**3.12.5.1 EBV LMP-1 Primer sequence: (**Appendix III)

Forward 5'-CCGAAGAGGTTGAAAACAAA-3'

Reverse 5'-GTGGGGGTCGTCATCATCTC -3'

**3.12.5.2 EBV EBNA-4 Primer sequence:** (Appendix III)

Forward 5'-GAGGAGGAAGACAAGAGTGG-3'

Reverse 5'-GATTCAGGCGTGGTCCTTGG-3'

(Yahia *et al*., 2014).

**3.12.5.3 PCR mix per sample:**

Maxime PCR PreMix Kit (iNtRON, korea).(Appendix II)

25

EBV LMP-1 forward primer 0.5µl

EBV LMP-1 reverse primer 0.5µl

EBV EBNA-4 forward primer 0.5µl

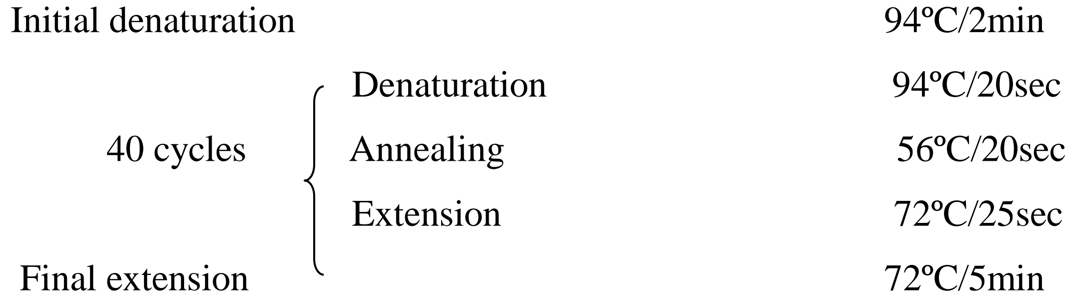
EBV EBNA-4 reverse primer 0.5µl

Sample DNA template 4µl

$dH_2O$ 19µl

Total volume 25µl

#### 3.12.5.4 PCR program:



| | | |
|---|---|---|
| Initial denaturation | | 94ºC/2min |
| 40 cycles | Denaturation | 94ºC/20sec |
| | Annealing | 56ºC/20sec |
| | Extension | 72ºC/25sec |
| Final extension | | 72ºC/5min |

### 3.12.6 Gel electrophoreses of the PCR products:

Ten microliterof the PCR products were loaded in 2% agarose (Appendix III).

#### 3.12.6.1 Agarose gel electrophoresis:

Agarose gel 2% was used for EBV LMP-1 (diagnostic band 161bp), and EBV EBNA-4(diagnostic band230bp). 10 microliters of each PCR products was loaded on agarose gel in the tank submerged with loading buffer (Appendix III) and then run at 100 volt for 15 minutes then at 75 volt for 10 minutes. For each gel a DNA ladder marker (iNtRON, korea), positive control and a negative controls were loaded and then followed by the samples. The gel transferred into a gel documentation system, to be viewed under UV light and photographed. A PCR results were regarded as positive if it has the same size as the expected size of the diagnostic band as read by the ladder marker and in comparison to the positive control. The positive controls were used to be compared with PCR product and to insure efficiency of PCR kits.

### 3.13 Quality control

Standard procedures for preventing contamination were strictly applied. New gloves for each sample and step and clean lab coat was used. DNA samples and

PCR products were kept in separate boxes. The PCR kits were divided into three separate sets (aliquots). Ice block was used during preparation of PCR mix inside sterile lamina flow hood. Distilled water used each time and were kept closed and frozen until needed .All pipette tips and Eppendorf tubes were autoclaved. No mobiles or chat with colleagues during PCR. The condition of each experiment was written and each experiment was photographed and the results were recorded. The bench and the hood were cleaned by ethanol before and after work. Each DNA sample and PCR product were fully labeled using water proof permanent fine tip black marker (sample number, PCR reaction and date).

### 3.14 Data analysis:

Collected data were analyzed using the application of statistical package for social science (SPSS) version 11.5. by using Chi square *p* value less than 0.05 was considered significant for the association between variables.

# CHAPTER FOUR
# RESULTS

Data presented in this study were obtained from previous records of laboratory investigations.

## 4.1Patients:

Formalin embedded tissue blocks (no=51) of Sudanese women with histologically confirmed breast cancer were selected. Their mean and age range were 51.18 and 59 respectively, 29/51 (57%) of patients were below 51 years, and 22/51 (43%) their ages were above 52 years.

Types of breast cancer for these patients were mostly invasive ductal carcinoma 48/51 (94%), ductal carcinoma in situ 1/51(2%), mucinous 1/51(2%), and infiltrating lobular 1/51(2%). Fig (4). The percentage of grade I in all patients is 4/51 (8%), grade II 32/51 (62%) and grade III 15/51 (30%).

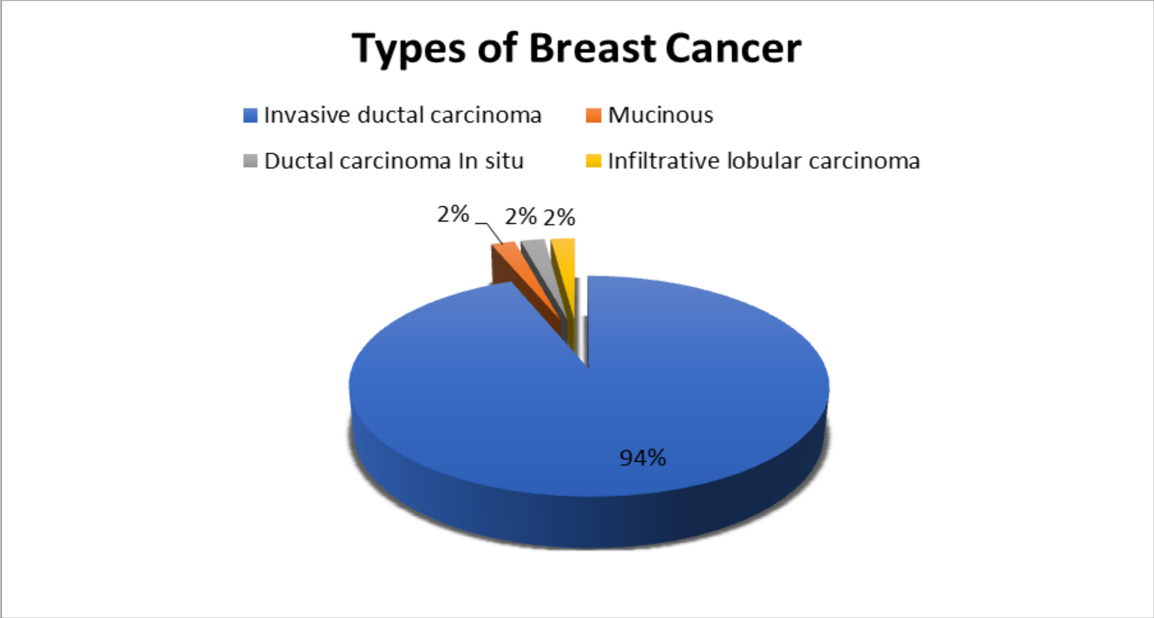



**Fig 4: Types of breast carcinoma conducted in the study.**

**4.2 Detection of EBV by PCR:**

Epstein Barr virus type specific PCR primers were used to amplify and identify EBV LMP-1 and EBV EBNA-4. The diagnostic band for LMP-1 (161bp) and diagnostic band for EBNA-4 (230bp). High annealing temperature was used to insure specific amplification of EBV LMP-1 and EBV EBNA-4 sequences, EBV other genes not amplified.EBV was identified in five samples (9.8%).

Results of this study showed in tables (1), (2), (3) and fig (5).

The 5\51 positive samples (9.8%) were detected in invasive ductal carcinoma but not detected in other types as EBV LMP-1 gene. Table (1).

**Table 1: Association between EBV and types of breast cancer:**

| | | | CANCER | | | | |
|---|---|---|---|---|---|---|---|
| | | | Invasive ductal | Infiltrative lobular | Ductal carcinoma in situ | Mucinous | Total |
| EBV | LMP-1 | Count | 5 | 0 | 0 | 0 | 5 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | % of Total | 9.8% | .0% | .0% | .0% | 9.8% |
| | EBNA-4 | Count | 0 | 0 | 0 | 0 | 0 |
| | | % of Total | 0% | .0% | .0% | .0% | 2.0% |
| | Non detected | Count | 43 | 1 | 1 | 1 | 46 |
| | | % of Total | 84.3% | 2.0% | 2.0% | 2.0% | 90.2% |
| Total | | Count | 48 | 1 | 1 | 1 | 51 |
| | | % of Total | 94.1% | 2.0% | 2.0% | 2.0% | 100.0% |

***P*** value 0.999

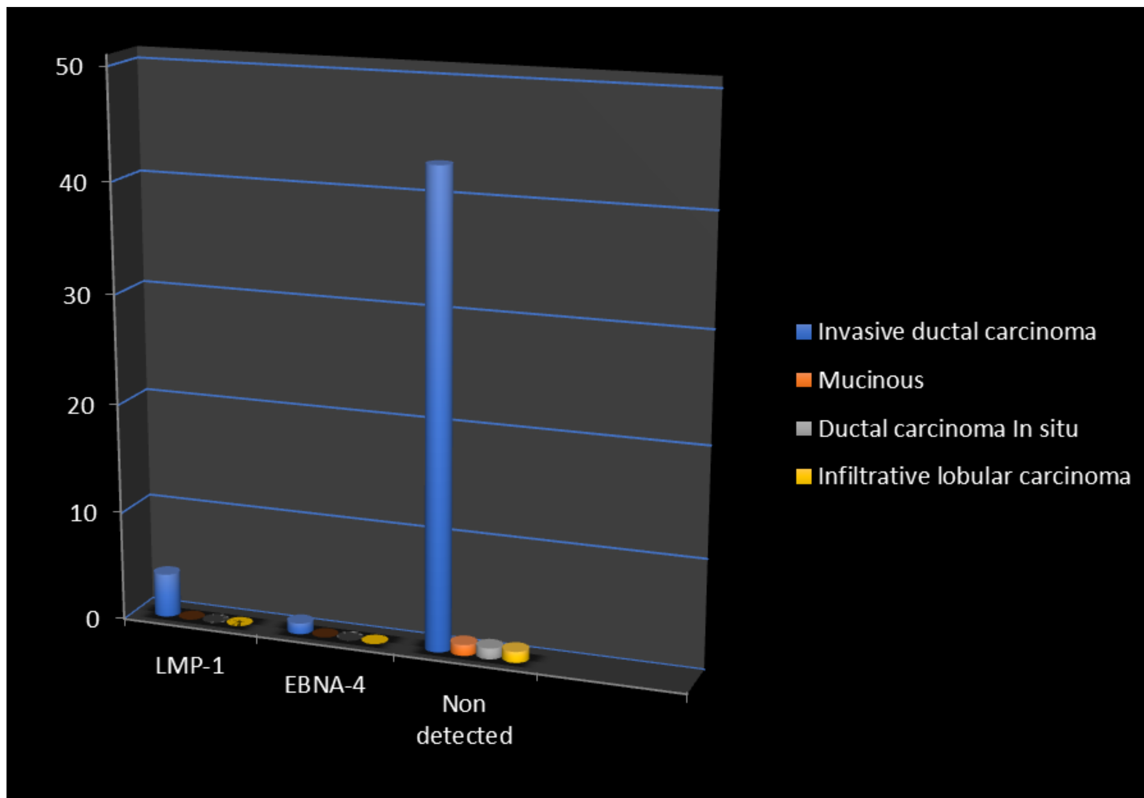



**Fig (5): Association between EBV and types of breast cancer**

Epstein Barr virus LMP-1 in undifferentiated cell detected in 1\51 (2%), poorly differentiated 2\51 (3.9%), and in well differentiated 2\51 (3.9%). While EBV EBNA-4 was not detected, Table (2).

**Table 2: Association of EBV and degree of cell differentiation:**

| | | D.O.D | | | Total |
|---|---|---|---|---|---|
| | | undifferentiated | poorly differentiated | well differentiated | |
| EBV | LMP-1 | Count | 1 | 2 | 2 | 5 |
| | | % of Total | 2.0% | 3.9% | 3.9% | 9.8% |
| | EBNA-4 | Count | 0 | 0 | 0 | 0 |
| | | % of Total | .0% | .0% | .0% | .0% |
| | Non detected | Count | 4 | 29 | 13 | 46 |
| | | % of Total | 7.8% | 56.9% | 25.5% | 90.2% |
| Total | | Count | 5 | 31 | 15 | 51 |
| | | % of Total | 9.8% | 60.8% | 29.4% | 100.0% |

***P-* value**: 0.468

In the age groups 36-45 and 46-55 2 EBV were detected in each of them while one EBV was detected in age group 66-75. Table (3).

**Table 3: Show association of EBV with age groups of participant:**

| | | | Age | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | | | 25-35 | 36-45 | 46-55 | 56-65 | 66-75 | 76-85 | |
| EBV | LMP-1 | Count | 0 | 2 | 2 | 0 | 1 | 0 | 5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | % of Total | .0% | 2.0% | 3.9% | .0% | 2.0% | .0% | 9.8% |
| EBNA-4 | Count | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | % of Total | .0% | .0% | .0% | .0% | .0% | .0% | 2.0% |
| Non detected | Count | 7 | 9 | 14 | 11 | 3 | 2 | 46 |
| | % of Total | 13.7% | 17.6% | 27.5% | 21.6% | 5.9% | 3.9% | 90.2% |
| Total | Count | 7 | 11 | 16 | 11 | 4 | 2 | 51 |
| | % of Total | 13.7% | 21.6% | 31.4% | 21.6% | 7.8% | 3.9% | 100.0% |

***P*-value**: 0.669

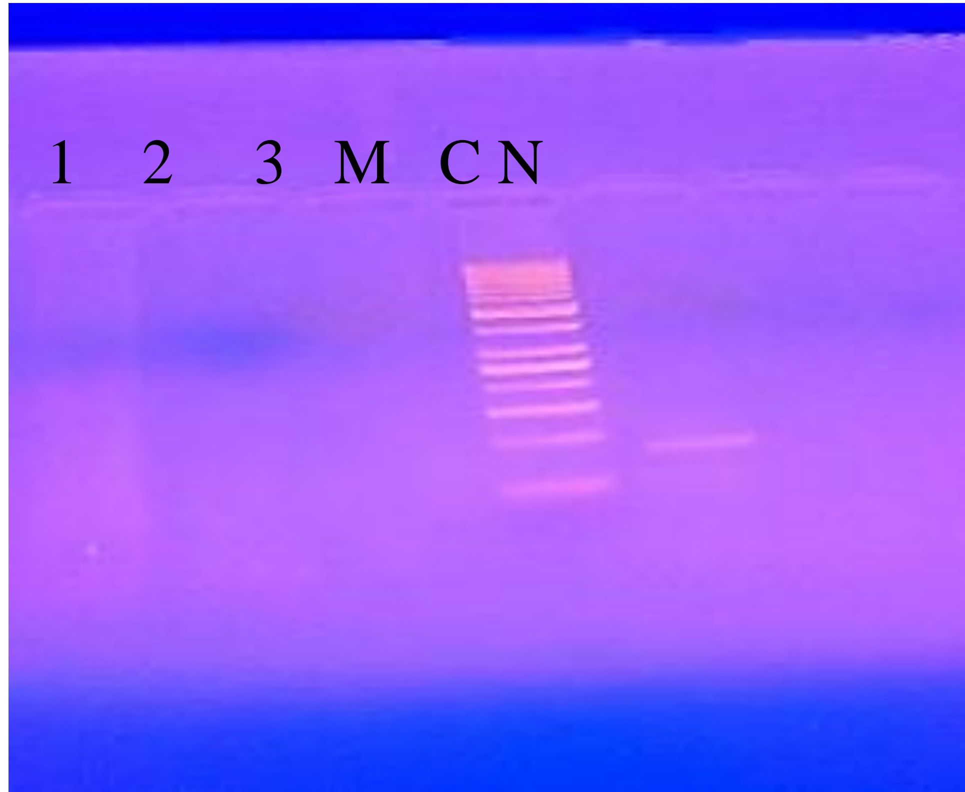



**Figure (6) Agarose gel electrophoresis of multiplex PCR product, Lane M (Marker): 50bp ladder, Lane C control positive for EBV LMP-1 gene, N negative control, Lane 1, 2, 3 negative samples.**

**Figure (7) Agarose gel electrophoresis of multiplex PCR product, Lane M (Marker): 100bp ladder, Lane C control positive for EBV LMP-1 gene, N negative control, Lane 1, 2, 3, 4 negative samples, lane 5 positive sample for EBV LMP-1 gene.**

# CHAPTER FIVE

# DISCUSSION

## 5.1 Discussion

The number of women with breast cancer increase annually. The association between EBV and breast cancer has now been assessed in various studies.

High risk of EBV were approved to be an aetiological agent for breast cancer by many scientists. This study aimed to detect and identify EBV that may be present in paraffin embedded sections of breast cancer tissues.

The overall positivity for EBV in this research was 5/51 (9.8%). EBV detected in women with invasive ductal carcinoma (9.8%) and (0%) patients by LMP-1 and EBNA-4 PCR respectively. And not detected in cases of infiltrative lobular, ductal carcinoma *in situ*, and mucinous carcinoma.

EBV detected in undifferentiated tumor (2%), poorly differentiated (3.9%), and in well differentiated (3.9%) by LMP-1 and not detected by EBNA-4.

In this study we found no significant association of EBV presence and age of women ($p > 0.05$).

However, the positivity for EBV that was reported before by many authors was (53%) and (11%) patients by LMP-1 and EBNA-4 PCR respectively (Yahia *et al*., 2014). By (Zekri *et al.,* 2012) EBV was detected in 18\40 (45%) and 14\50 (28%) of Egyptian and Iragi women.

Labrecque et al (1995) found that 21% of breast cancer tissues were found positive to EBV while luqmani and shousha (1995) detect 54% positive using PCR.

Some studies have reported incidence in breast cancer tissue as high as 21-51% (Thompson and Kurzrock, 2004). Bonnet et al (1999) detected EBV genome by PCR in 51% of tumors, whereas, in 90% of cases studied the virus was not detected in healthy tissue adjacent to the tumor ($P<.001$).

The low positivity in this study may be due to the fact that different PCR primers and diverse molecular techniques were used and their sensitivity and specificity are different. Some authors used EBV general primers and applied low annealing temperature to increase the sensitivity but decrease the specificity eg: (Labrecque *et al*., 1995), this may lead to false positive results due to non specific amplification. Some of those authors combined two or more techniques PCR,

hybridization, and enzyme immune assays to enhance their sensitivity and specificity eg: Yahia *et al*., 2014 and Bonnet *et al*., 1999. The storage condition of PCR reagents may decrease their efficiency and sensitivity.

Other Possible reasons for differences may include: differing EBV derived proteins or RNAs analyzed, and epidemiological variation in EBV infections or in breast cancer itself. Regardless, whether EBV is present in breast cancer and its possible etiological role in oncogenesis association should be clarified (Thompson and kurzrock, 2004).

## 5.2 CONCLUSION

In this study EBV was only detected in invasive ductal carcinoma but not in infiltrative lobular, ductal carcinoma *in situ*, and mucinous carcinoma. It is also concluded that EBV LMP-1gene was only detected. There was no significant association between virus detection and type of carcinoma and type of cell differentiation.

The virus was detected in age group 36-45 and 66-75 year.

## 5.3 Recommendations

1. Further study with large sample size and involving all part of the Sudan is recommended to get conclusive results on EBV infection.
2. We recommend the establishment of a national breast cancer registry system. Important for the control and prevention of breast cancer.

3. Increase awareness about the risk of EBV transmission, disease, and vaccination.
4. Validating assay sensitivity and specificity before interpreting results on EBV relatedness.
5. Convincing positive results of analytically well-conducted studies would support further work to characterize the epidemiology of EBV-related breast cancer and identify the high-risk patient subsets, to examine the possible interaction of EBV presence with biomarkers like hormone receptor status and markers of immune function, and to understand the role of EBV in development or maintenance of cancer cells.

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# Appendix I
# Lab Instruments

- Autoclave: used for sterilization.
- Incubater: incubation of tissues after adding protinase K.
- Sensitive balance: weight chemicals and reagents.
- Distiller: production of distilled water.
- Refrigerator and deep freezer: preservation samples and primers
- Centrifuge: for centrifugation.
- Pipettes.
- Microwave: dissolve the agarose powder.
- Casting tray: loading the gel.
- Voltage Source.
- Documentation system: provide the UV light.

# Appendix II
# Chemicals and reagents

- Xylene.
- Ethanol (100%,80%,70%,50%).
- d$H_2O$.
- Nucleic lysis buffer.
- Protinase K.
- RNase.
- NaCl (saturated)
- Primers (LMP-1&EBNA-4)
- Maxime PCR PreMix Kits.*
- Ethidium bromide.
- 10X Tris Boric EDTA buffer (TBE buffer).
- 1X Tris Boric EDTA buffer.
- TE buffer.
- DNA ladder marker.

*** Maxime PCR PreMix ( *I*- Taq):**

| Component in | 20µl reaction |
|---|---|
| *i-Taq*™ DNA Polymerase(5U/µl) | 2.5U |
| dNTPs | 2.5mM each |
| Reaction buffer(10x) | 1x |
| Gel loading buffer | 1x |

# Appendix III
# Preparations of Solutions

**(1) Ethanol:** 500 ml

| | |
|---|---|
| 500 ml ethanol ( ice cold or -20℃ ) | 100% |
| 400 ml ethanol + 100 ml $dH_2O$ | 80% |
| 350 ml ethanol + 150 ml $dH_2O$ | 70% |
| 250 ml ethanol + 250 ml $dH_2O$ | 50% |

**(2) Nucleic Acid Lysis buffer:** 100 ml $dH_2O$

| | |
|---|---|
| Tris Base ( MW. 12.14g ) | 0.12g |
| NaCl ( MW. 58.44g ) | 2.3g |
| $Na_2EDTA$ ( MW. 74.4g ) | 0.07g |
| SDS | 0.7g |

**(3) NaCl (saturated 6M):** 100 ml $dH_2O$

NaCl ( MW. 58.44g ) 35.1g

**(4) 10X Tris Boric EDTA buffer (TBE buffer):** 1 liter $dH_2O$

| | |
|---|---|
| Tris Base (MW. 12.14g ) | 48.4g |
| Boric Acid (MW) | 55g |
| $Na_2EDTA$ ( MW. 74.4g ) | 7.44g |

**1X TBE buffer**:

10 ml 10X TBE + 90 ml $dH_2O$.

**(5) Agarose Gel 2%:**

1g from agarose powder was measured and dissolved in 50 ml from 1X TBE buffer then microwaved for 2 min until agarose completely dissolved. Then was cooled to 55°C. 2µl from (10mg\ml) ethidium bromide were added and mixed well. Then the agarose was poured into gel tray with the well comb in place. Any bubbles were removed and the gel placed at room temperature for 20-30 min, until completely solidified. Then the comb was gently removed and the spacer from the opened sides was removed (Jalali et al., 2015).

**(6) loading buffer:**

1X TBE buffer 50 ml.

**(7)preparation of ethidium bromide solution:**

Ten milligram of ethidium bromide powder were dissolved into 500µl of deionized water, and kept into brown bottle.

**(8) Primers ( LMP-1&EBNA-4 forward and reverse):**

The stock solutions were prepared by adding 300µl from $dH_2O$ then were pelleted at 14,000 rpm for 5 min and placed at 4 ℃ over night. The working solutions were prepared by adding 10µl from stock solutions and 90µl from $dH_2O$ then mixed and placed at -20℃.

# Appendix IV

## Extraction and isolation of DNA from paraffin embedded tissue protocol

GMB006 Genomic Medicine Biorepository **GMB**

### Extraction and Isolation of DNA from Paraffin-Embedded Tissue

**Extraction of core**

1. If you intend to isolate DNA from both normal and tumor tissue, one must first prepare an H&E stain from a top slide of the tissue in the block. This slide should be read by an experienced pathologist, and the normal/tumor regions marked accordingly. Once this is done, you can use the slide to identify the corresponding regions in the block. Depending on the aim of the project, it may also be acceptable to use any or all parts of the tissue in the block.
2. Using a 14G needle as your cutting tool, pierce the block in the region of interest, and cut out a 1 to 3 mm core by turning the needle in the block. The depth of the cut should be sufficient to completely pierce the tissue. If you are unable to use the coring method, and can use DNA extracted from any part of the tissue, you can cut 6-8 10 um sections on the microtome and use for DNA extraction. Remember to wipe the stage with alcohol and replace the blade after each block, in order to avoid cross contamination.
3. Carefully transfer the newly cut core or shavings into either a 2 ml polypropylene microcentrifuge tube, or a 15 ml polypropylene centrifuge tube. Label the tube appropriately.
4. Repeat the coring or cutting process as necessary. Place each core or shaving into a separate tube.
5. At a later time, it is recommended that you seal the block with melted paraffin.

**Removal of Paraffin**

*(Note: Xylene is a hazardous chemical. The step 6 must be done in a fume hood, and the resulting waste handled appropriately.)*

6. Treat the core via the following steps in 2.0 ml polypropylene microcentrifuge tubes (15 ml polypropylene centrifuge tubes work as well – adjust centrifugation accordingly):
   a. Xylene, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   b. Repeat
   c. 100% Ethanol, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   d. Repeat
   e. 80% Ethanol, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   f. Repeat
   g. 50% Ethanol, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   h. Repeat
7. Add 1 ml $H_2O$ and incubate at 4°C overnight.

**Digestion of Protein**

8. Pellet sample at 14,000 rpm for 10 minutes – aspirate supt.
9. Add 700 ul of Nucleic Acid Lysis buffer (NALB).
10. Add 50 ul of Proteinase K (@ 30 mg/ml).
11. Incubate for 24 hours at 65°C.
12. Add an additional 50 ul of Proteinase K (@ 30 mg/ml).

Protocol GMB006rB: 060205REW
Revised and approved 2010DEC29 by N. Prescott

13. Incubate for an additional 24 hours at 65°C.

**Precipitation & Isolation of DNA**

14. If the sample requires the elimination of endogenous RNA (recommended), add 10 ul of RNAse A (10mg/ml) and incubate at 37°C for 30 minutes.
15. Add 250 ul of 6 M NaCl (saturated).
16. Let stand at room temp for 10 minutes.
17. Pellet sample at 14,000 rpm for 10 minutes.
18. Carefully transfer supernatant to a clean microcentrifuge tube.
19. Add 1 ml of ice-cold (or –20°C) 100% Ethanol.
20. Carefully mix and place at –20°C for 20 minutes.
21. Pellet sample at 14,000 rpm for 10 minutes.
22. Carefully discard the supernatant.
23. Wash pellet with 1.5 ml of 70% Ethanol.
24. Pellet sample at 14,000 rpm for 10 minutes.
25. Carefully discard the supernatant.
26. Allow pellet to air dry on the benchtop for 10 to 15 minutes.
27. Add 30 to 80 ul of TE Buffer.

Nucleic Acid Lysis Buffer
10 mM Tris Base (1.21 g/L)
400 mM NaCl (32.4 g/L)
2 mM $Na_2EDTA$ (0.75 g/L)
0.7% SDS (7.0 g/L)

# Maxime PCR PreMix Kit

**Maxime PCR PreMix Series**

*Research Use Only*

ISO 9001/14001 Certified Company

## *Maxime* PCR PreMix Kit ( *i*-Taq )

**for 20μl rxn / 50μl rxn**

**Cat. No. 25025**(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
**Cat. No. 25035**(for 50μl rxn, 96 tubes)

### DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
*Maxime* PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

### STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

### CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

### CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20μl rxn) — 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50μl rxn) — 96 tubes

| Component in | 20 μl reaction | 50 μl reaction |
|---|---|---|
| *i-Taq*™ DNA Polymerase(5U/μl) | 2.5U | 5U |
| dNTPs | 2.5mM each | 2.5mM each |
| Reaction Buffer(10x) | 1x | 1x |
| Gel Loading buffer | 1x | 1x |

**Note :** The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

### PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   **Note 1 :** Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   **Note 2 :** Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl .
   Do not calculate the dried components

**Example** — **Total 20μl or 50μl reaction volume**

| PCR reaction mixture | Add | Add |
|---|---|---|
| Template DNA | 1 ~ 2μl | 2 ~ 4μl |
| Primer (F : 10pmol/μl) | 1μl | 2 ~ 2.5μl |
| Primer (R : 10pmol/μl) | 1μl | 2 ~ 2.5μl |
| Distilled Water | 16 ~ 17μl | 44 ~ 41μl |
| **Total reaction volume** | **20 μl** | **50 μl** |

**Note :** This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   **Note** : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   **Note** : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

### SUGGESTED CYCLING PARAMETERS

| PCR cycle | | Temp. | PCR product size | | |
|---|---|---|---|---|---|
| | | | 100-500bp | 500-1000bp | 1Kb-5Kb |
| Initial denaturation | | 94℃ | 2min | 2min | 2min |
| 30-40 Cycles | Denaturation | 94℃ | 20sec | 20sec | 20sec |
| | Annealing | 50-65℃ | 10sec | 10sec | 20sec |
| | Extension | 65-72℃ | 20-30sec | 40-50sec | 1min/Kb |
| Final extension | | 72 ℃ | Optional. Normally, 2-5min | | |

### EXPERIMENTAL INFORMATION

- *Comparison with different company kit*

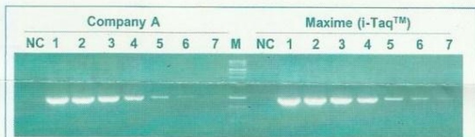


**Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment .**
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
**Lane M,** SiZer-1000 DNA Marker; **lane 1,** undiluted λDNA; **lane 2,** 200 ng λDNA; **lane 3,** 40 ng λDNA; **lane 4,** 8 ng λDNA; **lane 5,** 1.6 ng λDNA; ; **lane 6,** 320 pg λDNA; **lane 7,** 64 pg λDNA; **lane NC**, Negative control

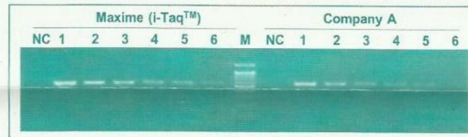


**Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).**
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
**lane M,** SiZer-100 DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA; **lane 6,** 1/32 diluted cDNA; **lane NC**, Negative control


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