THE ASSOCIATION OF EPSTEIN–BARR VIRUS INFECTION WITH PROGRESS OF CANCER AND IMMUNE DEFECTS

Soran Shareef SABER

MASTER THESIS

Department of Biology

Supervisor: Prof. Dr. Viktor NEDZVETSKYI

2017

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REPUBLIC OF TURKEY
BİNGÖL UNIVERSITY
INSTITUTE OF SCIENCE

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PREFACE

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LIST OF SYMBOLS

AP	: Activating Protein
APC	: Antigen presenting cells
AIDS	: Acquired immunodedicincy syndrome
AN	: Alkaline nuclease
BAK	: BCL2 Antagonist killer
Bax	: BCL2 Associated X
Bad	: BCL2 Associated death
BARF-1,2	: Bam HI-A region fragment-1,2
BARTs	: Bam HI-A region transcripts
Bbc3	: BCL2 Binding component 3
BCL-2	: B-cell lymphoma 2
BCL-xl	: BCL extra large
BCR	: B cell receptor
bf1	: BCL fetal liver
BH	: BCL-2 Homoglogy
Bid	: BH3 interacting death
BIK	: BCL2 interacting killer
BL	: Burkitt’s Lymhoma
BOD	: BCL2 related ovarian death

BOK	: BCL2 related ovarian killer
BUMA	: P53 upregulated modulator of apoptosis
CBC	: Complete blood count
CBF1	: C promoter binding factor
CD	: Cluster of differentiation
CLIA	: Chemiluminescence immunoassay
CMV	: Cytomegalovirus
CNV	: Central nervous system
CR	: Cellular receptor
CSTs	: Complementary strand transcripts
CTAR2	: Carboxyl terminal activate region 2
DW	: Distilled water
DNA	: Deoxyribonucleic acid
EA	: Early antigen
EBNA	: EBV nuclear antigen
EBERs	: EBV-encoded small nonpolyadenylated RNAs
EDTA	: Ethylene diamine tetraacetic acid
EIAs	: Enzyme immunoassay
ELISA	: Enzyme-linking immunosorbent assay
GC	: Gastric carcinoma
gp	: Glycoprotein
gH,L	: Glycoprotein H, L
HIV	: Human immunodeficiency virus
HLA	: Human leukocyte antigen

IFAs	: Immunofluorescence assays
IgA,G,M	: Immunoglobulin A,G,M
IHC	: Immunohistochemistry
IL	: Interleukin
ISH	: In situ hybridization
L	: Ribosomal Protein
LA	: Late antigen
LCLs	: Lymphoblastoid cell lines
LMP	: Latent membrane protein
LP	: Leader protein
MA	: Membrane antigen
MCL	: Myeloid cell leukemia
MCL1	: Induced myeloid leukemia cell
MFI	: Multiplex flow immunoassay
MHC	: Majer histocompatibility complex
mRNA	: Messenger RNA
MIR	: MicroRNA
miRNA	: MicroRNA
NC	: Nasopharyngeal Carcinoma
NCAM	: Neural cell adhesion molecule
NK	: Natural killer
NKT	: Natural killer T
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction

PKR	: RNA dependent protein kinase
PTLD	: Post-transplant lymphoproliferative disease
PAGE	: Polyacrylamide gel electrophoresis
RIPA	: Radioimmunoprecipitation assay
sisRNAs	: Stable intronic equence RNAs
SLE	: System Lupus Erythematosus
SDS	: Sodium dodecyl sulfate
snoRNAs	: Small nucleolar RNAs
TBS-T	: Tris Bass Solution- Tween
TCR	: T cell receptor
TNFR	: Tumor necrosis factor receptor
VCA	: Viral capsid antigen
WHO	: World health organization

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EPSTEIN–BARR VİRÜS ENFEKSİYONUNUN BAĞIŞIKLIK SORUNLARI VE KANSER GELİŞİMİYLE İLİŞKİSİ

ÖZET

Epstein-Barr (EBV) virüsü doğrudan karsinogenezle ilişkilendirilen ilk insan virüsüdür. Dünyada insan populasyonunun % 90'ını enfekte eden bir virüstür. EBV herpes virus ailesindendir. EBV infeksiyonları hem litik hem de uzun süren latent enfeksiyon oluşturmaktadır. EBV virüsü B lenfositlerin dahil olduğu bağışıklık mekanizmasından kaçar ve sinyal yolağını kontrol eder. EBV yayılması ile alakalı önemli bir teşhis bağışıklık hücrelerinin özellikle doğal öldürücü hücrelerinin reaksiyon göstermesidir. Bu çalışmanın amacı bağışıklık tepkisinin bozulmasını arttıran moleküler mekanizmaların karsinojenezle arasındaki ilişkiyi ortaya çıkarmaktır. Bu çalışmada EBV infeksiyonu ile kanser gelişimi ve bağışıklık sorunları arasındaki ilişkiyi belirlemek için hastalardan alınan kan örnekleri kullanılmıştır. Örnekler Süleymani şehrinde yaşayan kanser hastalarından Hiwa hastanesinden toplanmıştır. Nazofarinks, gastric ve göğüs kanseri hastalarından toplamda otuz tane limfosit ve monosit örnekleri elde edilmiştir. Sağlıklı bireylerden alınan örnekler kontrol olarak kullanılmıştır.

EBV ve checked EBV- encoded kodlama yapmayan RNA varlığını belirlemek için standart PCR ve real-time PCR (RT-PCR) yöntemleri kullanılmıştır. Western blotlama ile CD56 ekspresyonunu ve tüm kan sayımını göstermek için CD56 antikoru kullanılmıştır. PCR analiz sonuçları ile elde edilen sonuçlar, bireylerin %90'dan fazlasının EBV enfeksiyonu açısından pozitif olduğunu göstermiştir. Üstelik bu sonuçlar, hastalarda nazofaringal karsinom (%60), gastrik karsinom (%50) ve meme kanserinde (%35) latent enfeksiyon onkogeninin (EBER-2) pozitifliğinin sırasıyla gösterildiğini ortaya koymuştur. Western blotlama sonuçları, hasta gruplarının lökositlerindeki CD56 içeriğinin artmasının, kontrol numunelerine kıyasla meme kanseri (%82), nazofaringal karsinom (%39) ve gastrik karsinomanın (%45) var olduğunu gösterdi. HBC (%26), WBC (%15), MPV (%10), lenfositler (%50) ve Granülositler (%20) ile hastaların %80'den fazlasının bir sonraki Tam kan sayımı parametrelerinde azalma olduğunu gösteren kan hücrelerinin analizi Elde edilen sonuçlar, EBV enfeksiyonu ile kanserojenez arasında, özellikle de nazofarengeal karsinom ve gastrik karsinomun incelenen örnekleri için farklı kanser tipleri arasında ilişki olduğunu gösterdi. Çalışılan hasta grublarında EBER-2 onkogeninde her iki karsinoma türünde de (%50) oranında daha fazla pozitif sonuç elde edildi.

Anahtar Kelimeler: EBV, PCR, CD56, EBER-2, tam kan sayımı.

THE ASSOCIATION OF EPSTEIN –BARR VIRUS INFECTION WITH PROGRESS OF CANCER AND IMMUNE DEFECTS

ABSTRACT

Epstein-Barr virus was the first human virus to be directly implicated in carcinogenesis. It infects more than 90% of the world's human population. EBV is a member of the herpes virus family. EBV infection forms both a lytic or long-term latent infection. EBV escapes from immunologic mechanism with infection of B-lymphocytes and control different signaling pathways. The important data on prognosis EBV expansion is immune cells response, especially natural killer (NK) cells. The aim of this study is an elucidation of molecular mechanisms that induce the disturbance of immune response and cancerogenesis.The study to establish relation between the association of Epstein-Barr virus infection and the progress of cancer and immune defects performed with using the samples of blood patients. The samples were collected in the Hiwa hospital specialized for cancer disease in Sulaymaniyah city during the period from springtime-summer 2016. Thirty samples of lymphocytes and monocytes were collected from blood of patients with nasopharyngeal carcinoma, gastric carcinoma and breast cancer. Ten subjects that were age and gender matched with study groups as healthy control were used.

Both standard PCR and Real-time PCR (RT-PCR) were used to detect presence of EBV and checked EBV-encoded noncoding RNA as a functional backup of viral oncogene depending on conventional method. Western blot method was used for determination antibody against CD56 and complete blood count (CBC) for disorder characteristics. Obtained with PCR analyses results shown that more than 90% of the individuals were identified as a positive result of EBV infection. Moreover, these results demonstrated that positivity of Latent infection gene (EBER-2) oncogene in nasopharyngeal carcinoma (60%), gastric carcinoma (50%) and breast cancer (35%) in patients respectively. The results western blot shown the increasing of CD56 content in leukocytes of the patient groups have breast cancer (82%), gastric carcinoma (45%) and nasopharyngeal carcinoma (39%) compared with control samples. The analysis of blood cells evident that more than 80% of the patients have a decrease for next CBC parameters HGB (26%), WBC (15%), MPV (10%), Lymphocytes (50%) and Granulocytes (20%) related with control. Presented results let assume the association between EBV infection and cancer genesis for distinct types of cancer, especially, for studied samples of nasopharyngeal carcinoma and gastric carcinoma. There determined for both carcinomas about 50% and more positive results for EBER-2 oncogene in studied cohort of patients.

Key words: EBV, PCR, CD56, EBER-2 and complete blood count.

1. INTRODUCTION

Epstein-Barr virus was the primary individual virus to happen straight involved in carcinogenesis. This virus infects more than 90% of the world's population. Although greatest humans cohabit with the virus exclusive of severe result, a little percentage drives progress tumors. There are numerous ideas on how the developments and progressions of cancers occur. Several studies have clarified this process beside the usage of a multistep cancer development traditional (Ortmann et al. 2015). Epstein-Barr virus (EBV) is connected in a diversity of individual diseases. It is the causal agent of heterophile-positive infectious mononucleosis. EBV contagion is likewise correlated with cancers such as B-cell lymphoma, Hodgkin's disease, Burkitt's lymphoma, and nasopharyngeal carcinoma (Roizman 1990). Unlike other herpesviruses that rely on lytic duplication for spread inside a host, EBV relies more on its latent mechanism. The infection is typically constricted from an asymptomatic individual who sheds the virus. Later infecting the oropharyngeal epithelium and spreads through the bloodstream (Murray and young 2002). The virus infects B-lymphocytes and causes an increase in T-lymphocytes and results in expansion of lymphoid tissue. In immunocompromised patients beside declined T-cell function, the B cells remain to multiply and this multiplication may lead to neoplastic conversion (Sixbey et al. 1984). Nearly, all organ systems can be affected by EBV. Neurologic complications such as encephalitis, meningitis and Guillain-Barré syndromes have been described. Hepatitis, myocarditis and hematologic disorders are also known complications (Babcock et al. 1998).

Seldom death results were detected from splenic rupture, CNS complications and airway obstruction. A rare form of chronic EBV infection characterized by a high titer of viral DNA has been reported. Patients are ill for ≥6 months and have histologic mark of chief organ participation and he diagnosis is lowly and healing is not well distinct (Decker et al.1998).

EBV-associated Gastric Carcinoma (EBVaGC) is clear as a monoclonal proliferation of carcinoma cells with latent EBV infection, which can be established by in situ hybridization targeted at EBV-encoded small RNA (EBER). In EBVaGC, almost all cancer cells comprise EBV DNA sequences and the EBV replication sequences have uniform lengths, implying that the tumor can develop from a only from EBV-infected cells and that the EBV genome persists during malignant transformation and proliferation (Busson et al. 2004).

The Association between EBV infection and breast carcinoma has been established in western countries, few studies have been recounted from Asian states. The virus is shown to be an etiologic factor in a substantial part of breast cancers. There would be strong grounds for speeding up efforts to develop an EBV vaccine, since breast cancer is the most common tumor of women worldwide, with nearly 800000 new cases per year (Joshi et al. 2009).

Both in disease and health, the life cycle of the Epstein-Barr virus is considered by a continuous interplay with the host immune system. To continue successfully and create dormancy in memory B-cells, this virus has progressed different strategies to evade immune system acknowledgment. Mononucleosis infection is a self-limiting lymphoproliferative illness, whose symptoms are caused by the massive release of cytokines by stimulated CD8+ T cells. Despite the limited nature of this disease, the interface between the host and the virus leaves some traces lifelong, reaching from a different repertoire of virus-specific T cells with particular characteristics (Cesarman 2011).

The important data on prognosis EBV expansion is immune cells response related with natural killer (NK) cells and the number of NK cells has been shown though the detected of CD56 and correlate with blood virus load (Pernick 2015).

The present study was carried out to focus on the possible role of EBER-2 and CD56 in the progress of cancer and defect of immune system in patients with nasopharyngeal carcinoma, gastric carcinoma and breast cancer.

2. LITERATURE REVIEW

2.1. Epstein-Barr Virus (EBV)

The Epstein–Barr virus also named human herpes virus 4 (HHV-4) can be divided into two major types EBV 1 and EBV 2 surrounded by the membrane envelope. These have different EBNA-3 genes and vary in their transforming capabilities and revival ability. EBV type 1 is dominant in most parts of the earth, but both are widespread in Africa (Joshi et al. 2009) (Figure 2.1).

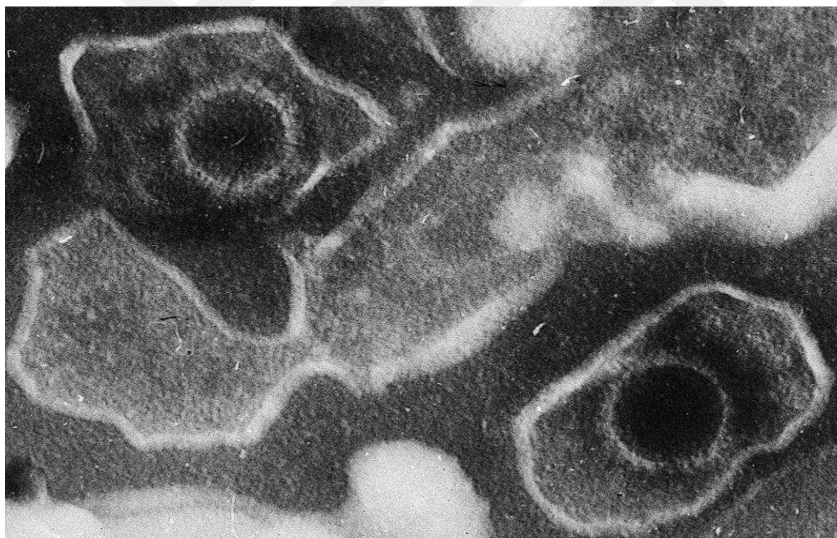

Figure 2.1. Two Epstein–Barr virions (Gross 2005)

2.1.1. Biological Background of EBV

The first associated of EBV infection with cancer was reported in 1964 and coincided with discovery by Epstein, Barr and Achong in electron micrographs of cells cultured from patient with endemic Burkitt's lymphoma (Leenman et al. 2004).

Epstein-Barr Virus is much more new and was classified as a group 1 carcinogen, an indication that there is the strongest possible evidence linking it to human cancer (WHO-IARC 1998).

2.1.2. Classification

Epstein-Barr Virus is a double stranded DNA virus, a member of Herpesvirales order, family Herpesviridae, sub-family gammaherpesvirinae and genus lymphocryptpvirus. The Herpesviridae family contain viruses grouped together based on the architecture of their virion (Roizman 1990).

2.1.3. Characteristcs

2.1.3.1. Structure and Genome

A-typical herpes virion consists of a core containing a linear, double helix DNA and an icosahedral capsid, approximately 122 - 180 nm in diameter, comprising 162 capsomeres with a hole running down the long axis; an amorphous, sometimes asymmetric material that surrounds the capsid, designated as the tegument and an envelope containing viral glycoprotein spikes on its surface, the virus about 172,000 base pairs in length and containing about 85 genes (Balfour 2012) (Figure 2.2).

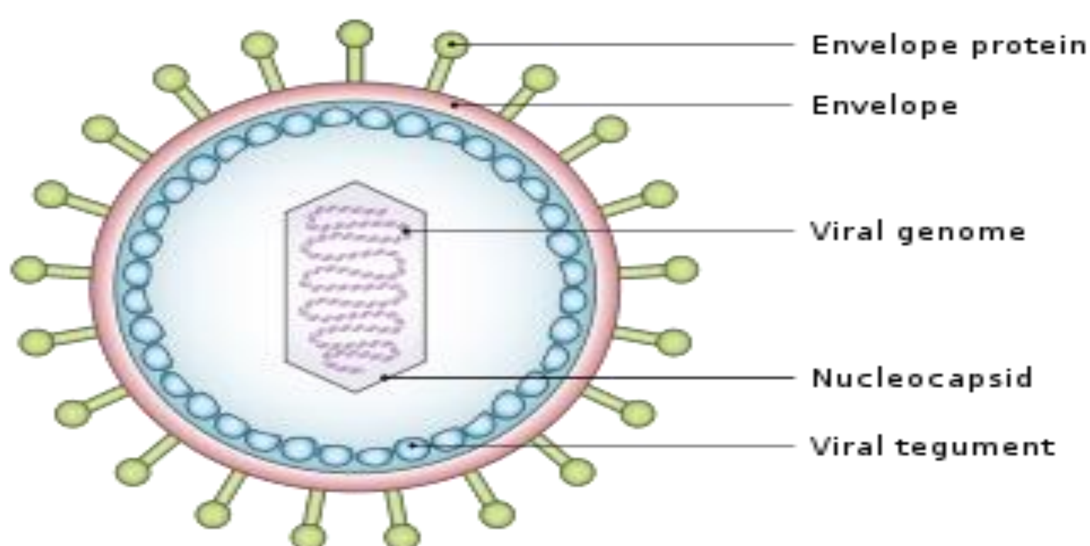

Figure 2.2. The structure of EBV (Taylor et al. 2010)

The collecting of analysis frames for related Epstein-Barr Nuclear Antigen (EBNA) and Latent membrane protein (LMP) were cleared through of the location of the origin of replication. The direction of transcription during latency III denotes and the direction of transcription for EBNA1indicates, which is activated during latency I, II. (Young and Rickinson 2004) (Figure 2.3).

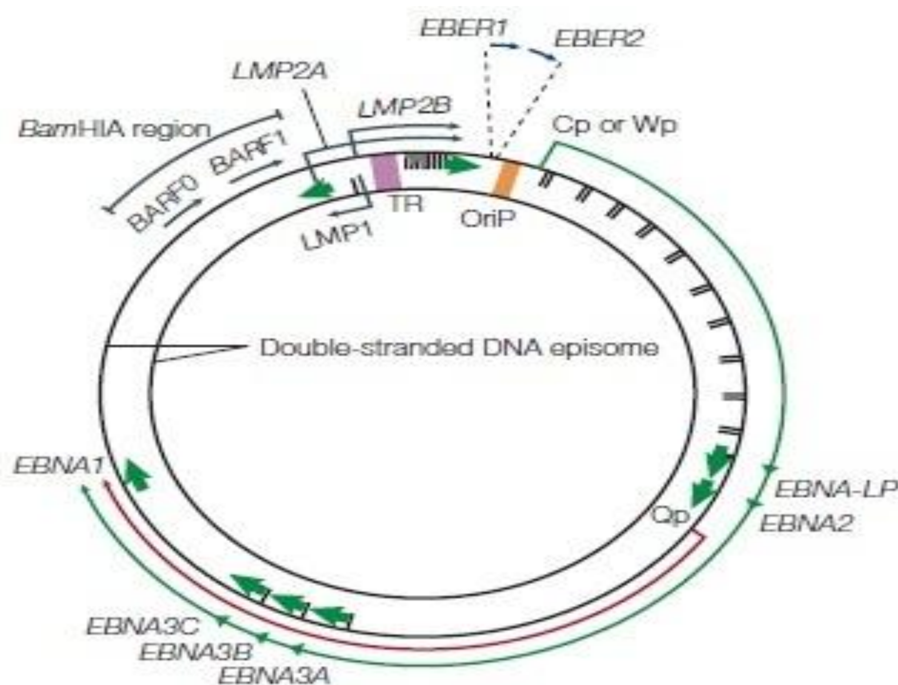

Figure 2.3. Epstein-Barr Virus dsDNA episome (Young and Rickinson 2004)

2.1.3.2. EBV of Tropism

Viral tropism means the cell kinds EBV infects. EBV is capable to infect diverse cell types containing B-cell and epithelial cells. The viral three-part glycoprotein multiplexes of gHgLgp42 umpire B-cell membrane fusion while the other complexes of gHgL intermediate epithelial cell membrane fusion. EBV ended In the B cells have little amounts of gHgLgp42 complexes, then these three-part multiplexes interrelate with Human-leukocyte-antigen class II molecules present and degraded in B-cells in the endoplasmic reticulum. Conversely, EBV from epithelial cells is rich in the three-part complexes so these cells do not usually have HLA class II molecules (Shannon and Rowe 2014).

2.1.4. Replication cycle

Epstein-Barr Virus is spread from host to host through salivary contact and the virus enters through the epithelium that lines the nasopharynx. After primary infection, the EBV genome becomes circular forming an episome in B-cells and remains latent in these cells (Cohen 2000) (Figure 2.4).

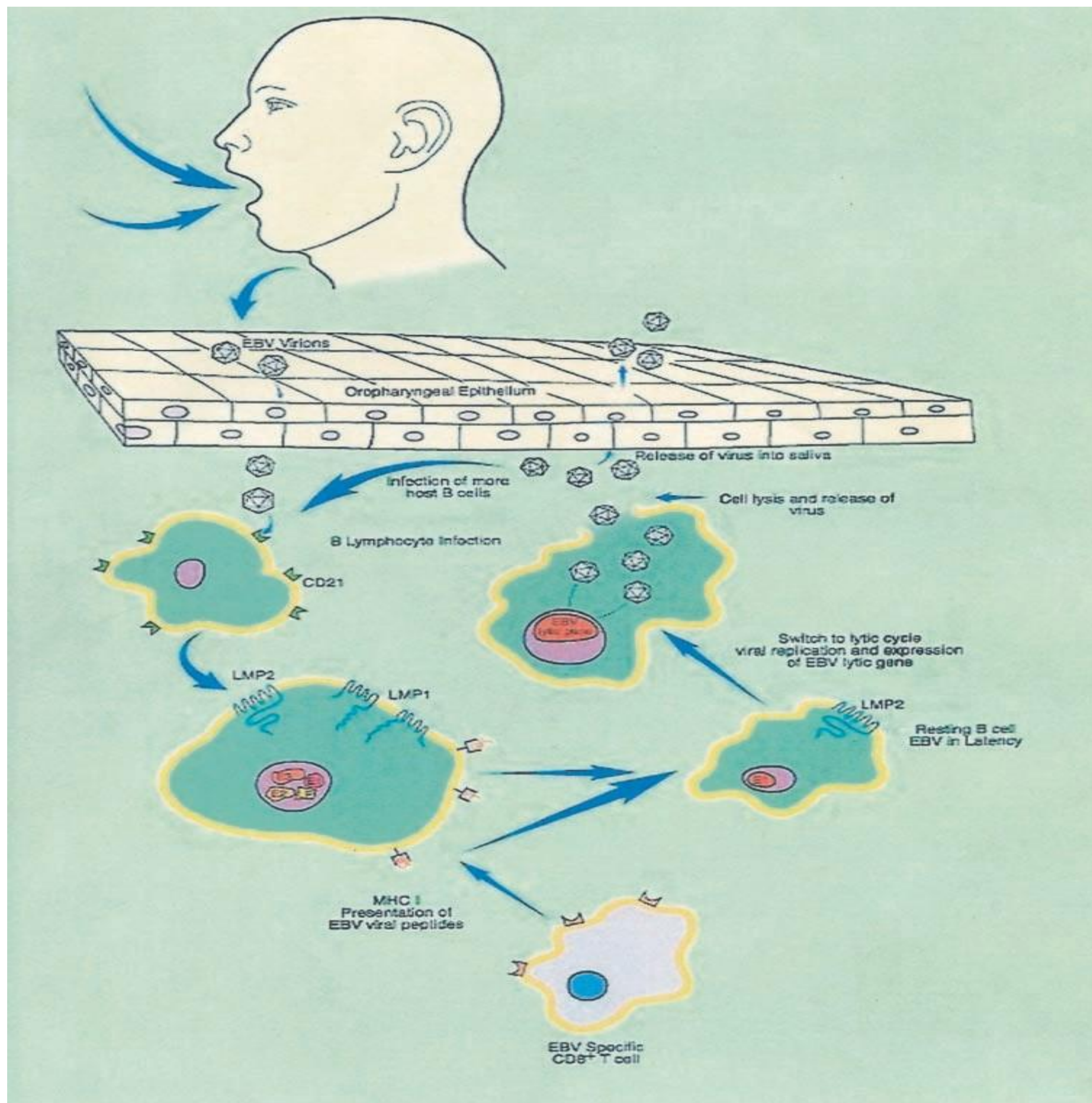

Figure 2.4. The life cycle of EBV (Thompson and Kurzrock 2004)

2.1.4.1. Entry to the Cell

A. B-Cells

Viral glycoprotein p350 binds to CD21 (CR2) cellular receptor named then viral glycoprotein gp42 interrelates with cellular MHC class II molecules. This activates fusion of the viral envelope with the cell membrane, allowing EBV to inter the B-cell. Human CD35 or called complement receptor 1(CR1) is an extra attachment factor for gp350/220, which provides a way for passage of EBV into CD21-negative immature B-cells (Ogembo et al. 2013) (Figure 2.5).

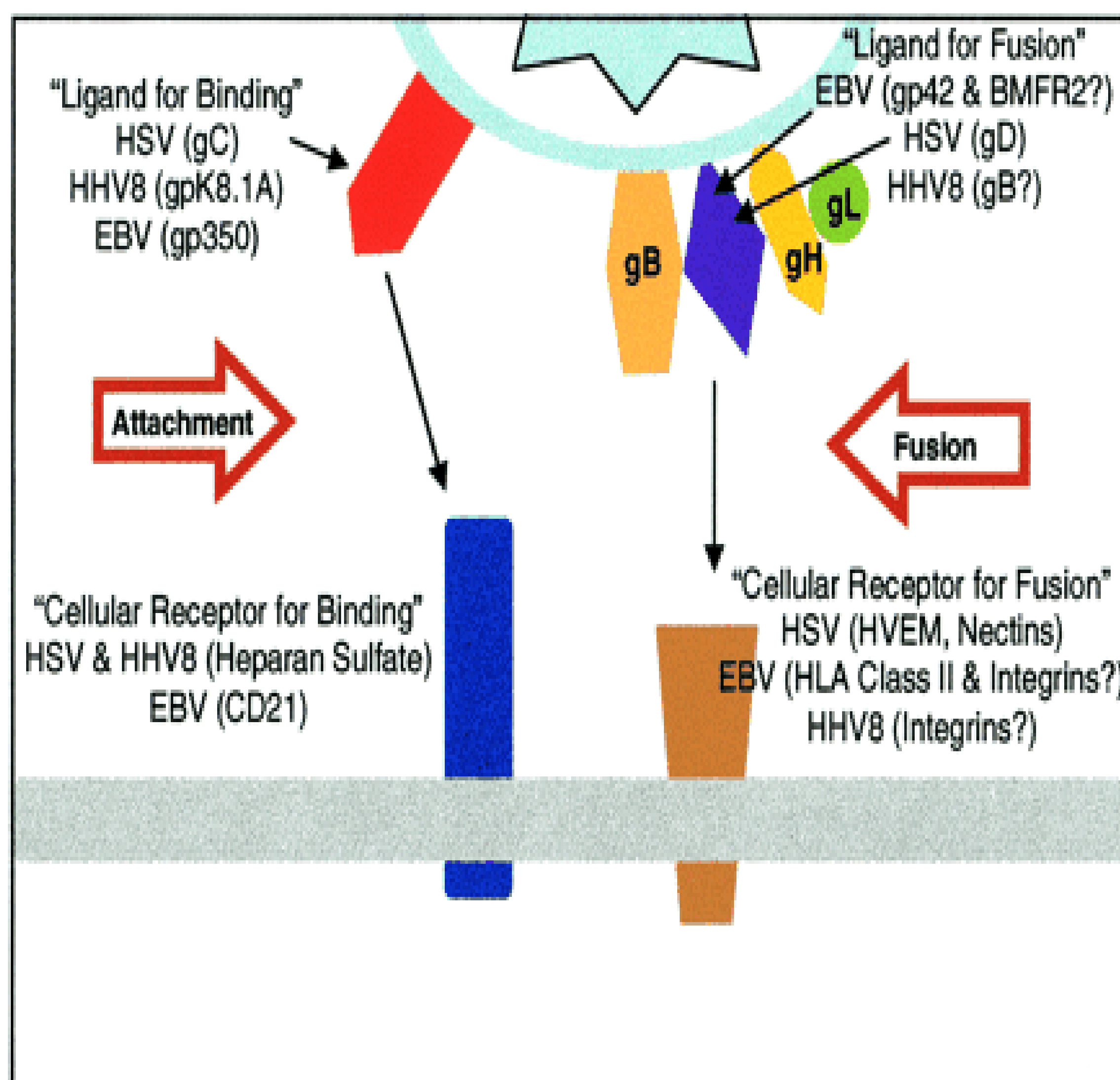

Figure 2.5. EBV enter to the B-cell (Wang and Hull 1998)

B. Epithelial Cells

The Epstein-Barr Virus BMRF-2 protein binds with cellular β1integrins and viral protein gH/gL interrelates with cellular αvβ6/αvβ8 integrins. This activates fusion of the viral envelope with the epithelial cell membrane that permition EBV entry into the epithelial cell. Epithelial cell entrance is inhibited by viral glycoprotein gp42. The host cell binding and fusion appear to be two distinct mechanisms, as fusion can occur without active binding ligands (Odumade et al. 2011).

2.1.4.2. Lytic Replication

Lytic cycle, also called productive infection, results in the production of infectious virions. EBV can suffer productive infection in B-cells and epithelial cells. In B-cells, productive infection mostly occurs following reactivation from latency but in epithelial cells a productive infection normally straight shadows viral entry Productive infection to occur. Latent EBV genome is circular which is needed to linearize in the process of lytic reactivation. During productive infection, DNA polymerase of virus is dependable to copy the viral genome but in latency, host cell DNA polymerase copies the genome of virus (Odumade et al. 2011).

2.1.4.3. Latency

The EBV genome circular DNA occurs in the cell nucleus as an episome next copied by cellular DNA polymerase. Only a portion of EBV's genes has gene expression. EBV exhibits one of three kinds of latency: Latency I, Latency II or Latency III. Each kind of latency leads to the manufacture of a partial, diverse group of viral proteins. It is also postulated that a program occurs in which the viral protein expression is shut off (Latency 0) (Lockey et al. 2008). Each latency program leads to the production of a limited, distinct set of viral proteins and viral RNAs as shown in Table 2.1.

Table 2.1. The Latency and Genes expressed by EBV (Amon 2004)

Gene Expressed	Product	Latency I	Latency II	Latency III
EBNA-1	Protein	+	+	+
EBNA-2	Protein	–	–	+
EBNA-3A	Protein	–	–	+
EBNA-3B	Protein	–	–	+
EBNA-3C	Protein	–	–	+
EBNA-LP	Protein	–	+	+
LMP-1	Protein	–	+	+
LMP-2A	Protein	–	+	+
LMP-2B	Protein	–	+	+
EBER	NcRNA	+	+	+

A. B-Cell

EBV latency in B cells ordinarily advances from Latency III. Later, the virus limits its gene expression and enters Latency II. Finally, EBV limits gene expression even extra and enters Latency I. The lymphoproliferative disorders associated with each type of expression (Amon 2004) (Figure 2.6).

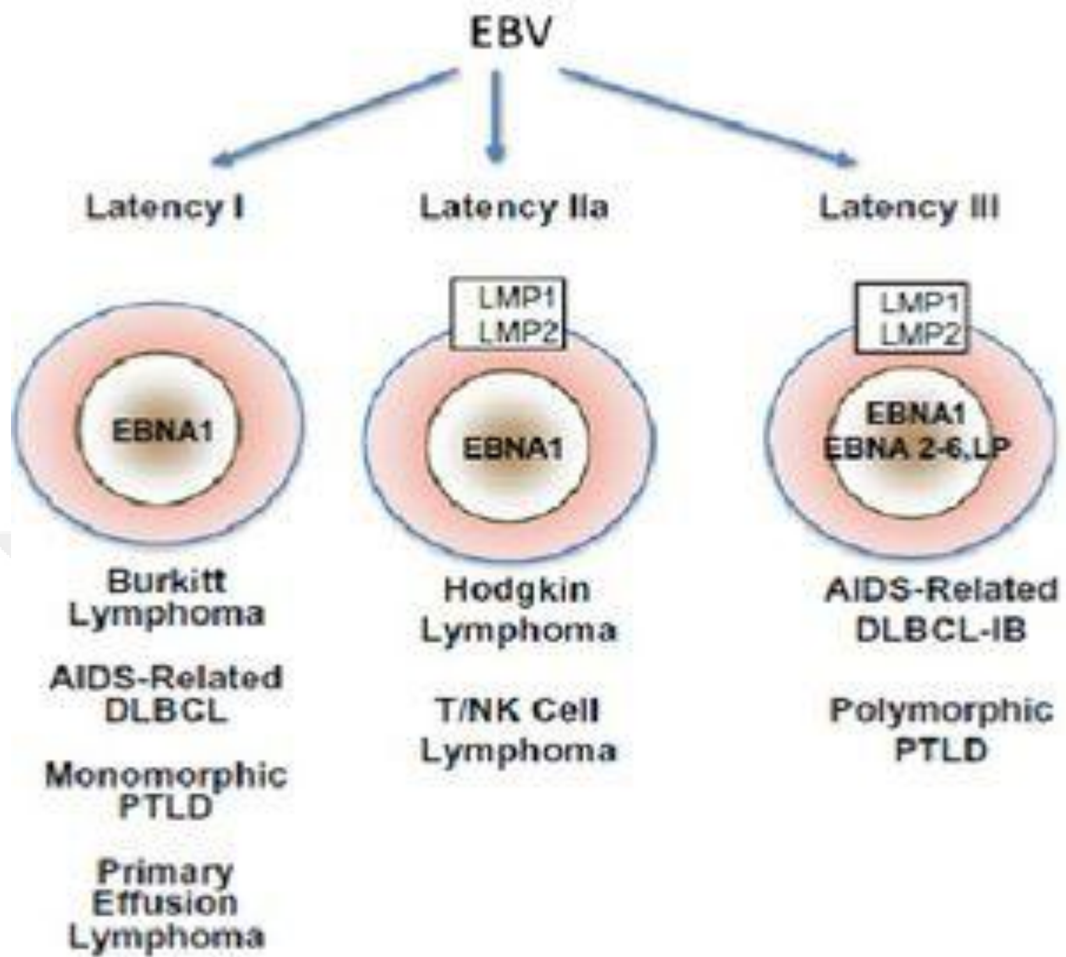

Figure 2.6. The Viral Latent Gene expression in EBV Infected B-Cells (Cesarman 2011)

B. Epithelial Cells

Only Latency II is possible in primary infection with EBV that replicates in oral pharyngeal epithelial cells, subsequent replication in epithelial cells can shed infectious virus into saliva. Latency II infection of NK- or T-cell is capable to result in malignancies (Hutzinger et al. 2009).

2.1.4.4. Reactivation

Latent EBV reactivation shifts to productive infection in vivo, but what activates it is not known exactly. Latent EBV can by reactivated by stimulating the B-cell receptor (BCR) (Odumade et al. 2011) (Figure 2.7).

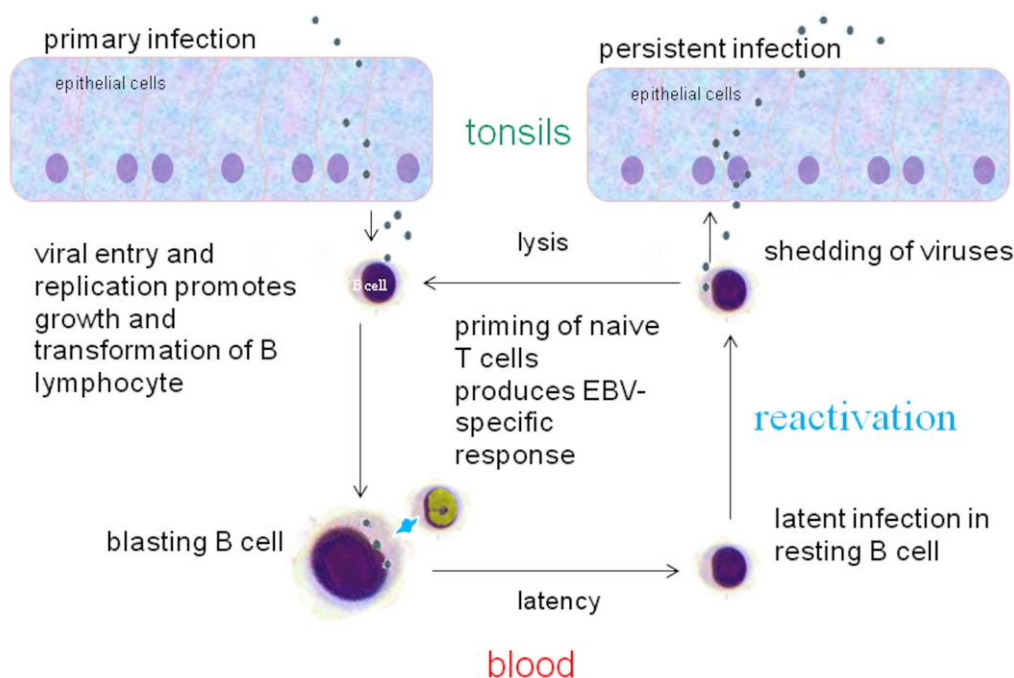

Figure 2.7. The Replication Cycle of EBV infection in healthy humans (Beards 2012)

2.1.5. Transformation of B-Lymphocytes

While EBV infects B-cells, lymphoblastoid cell lines eventually arise which is capable of unlimited growth. The lytic cycle produces large amounts of viruses to infect other B-lymphocytes within the host. The latency is reprogrammed and challenged infected B-lymphocytes to proliferate and transport infected cells to the sites at which the virus presumably persists. A balance is eventually uncovered between occasional viral reactivation and host immune surveillance eliminating cells that activate viral gene expression (Yates et al. 1985).

2.1.6. Epstein-Barr Virus Antigens

Epstein Barr Virus antigens are divided into three classes based on the phase of the viral life cycle in which they are expressed.

1- Latent phase antigens are synthesized by latently infected cell. These include the Epstein Barr Nuclear Antigens (EBNAs) and the Latent Membrane Protein (LMPs). Their expression reveals that an EBV genome present is only EBNA1, needed to maintain the viral DNA episomes, is invariably expressed; expression of the other latent phase antigens may be regulated different cells. LMP1 mimics an activated growth factor receptor.

2- Early antigens are non-structural proteins whose synthesis is not dependent on viral DNA replication. The expression of early antigens indicates the onset of product viral replication.

3- Lytic antigens are the structural components of the viral capsid (viral capsid antigen) and viral envelope (glycoproteins). They are produced abundantly in cells undergoing productive viral infection (Jawetz et al. 2007).

2.1.6.1. Epstein–Barr Nuclear Antigen-1 (EBNA-1)

Epstein-Barr Nuclear Antigens-1 in a stage of latent and lytic is expressed in all actively dividing EBV-infected cells and is responsible for binding the viral episome through its origin of replication (OriP) to the mitotic cellular DNA, assuring replication and transfer of virus genome to all daughter cells (Munz 2005).

EBNA-1 was also shown to be involved in the transcriptional control of other latency proteins, a function that is independent of episome maintenance unclear. It is the only viral protein expressed during group I latency. Almost the entire N- terminal half of the EBNA1 protein is composed of an irregular copolymer of the amino acids glycine and alanine that prevents EBNA1 from being degraded by the proteasome (Altmann et al. 2006).

2.1.6.2. Epstein-Barr Nuclear Antigen-2 (EBNA-2)

EBNA-2 is the viral protein mainly responsible for transactivating the major latency promoters Cp and LMP2Ap and a large number of cellular promoters involved in the process of B-cell immortalization. Thus, resulting in the morphological transformation of B-lymphocytes to immortalized LCLs. Viruses carrying an EBNA2 deletion are unable to transform B-cells. Promoter start by EBNA2 is through indirect binding to promoter DNA via interaction with a cellular protein, C promoter binding factor 1 (CBF1) (Cordier et al. 1990). Specific targets of EBNA-2 activation between several others are the promoters for the viral LMP1 gene and the cellular genes for the B-cell growth receptor CD23 and the EBV receptor CD21 (Wang 1996).

2.1.6.3. Epstein-Barr Nuclear Antigen-3 (EBNA-3)

The EBNA-3 family of proteins, EBNA-3A, EBNA-3B and EBNA-3C, has now been shown to have a function in modulating LMP-1 and LMP-2 transcription by preventing EBNA-2 transactivation of the LMP-1 and LMP-2 promoters (Longnecker and Miller 1996). EBNA3C has been shown to partially overlap the functions of EBNA-2, as it also regulates some viral and cellular genes that are regulated by EBNA-2, including CD21 and LMP-1. Like EBNA2, all three members of the EBNA3 protein family interact with promoter DNA of many genes through binding CBF1 and mostly negatively modulate the transcriptional effects of EBNA2 (Robertson et al. 1995).

2.1.6.4. Epstein-Barr Nuclear Antigens -Leader Protein

Epstein-Barr nuclear antigens -leader protein plays an important role in the establishment of B-cell immortalization by acting as an EBNA2 co-activator. This transcriptional activation preferentially enhances the expression of the major viral protein LMP1. It has been postulated that EBNA-LP may be important in regulation of EBNA expression or the regulation of virus or cell gene expression mediated by EBNAs. EBNA-LP is mRNA is transcribed across the internal repeat. Viruses with LP mutations do not efficiently transform B cells. So, LP is a transcriptional coactivator for EBNA2 and greatly increases immortalization efficiency (Longnecker et al. 2000).

2.1.6.5. Latent Membrane Protein-1 (LMP-1)

Latent membrane protein is a six-span transmembrane protein that is also essential for EBV-mediated growth transformation. LMP-1 is an integral plasma membrane protein associated with lipid rafts with a pattern of signaling that mimics constitutive CD40 triggering in a ligand-independent manner (Stunz et al. 2004).

Latent membrane protein-1 also induces the expression of cell surface adhesion molecules and activation antigens and up regulates anti-apoptotic proteins in B-cells. In epithelial cells, ectopic LMP-1 expression leads to hyper-proliferation, inhibition of differentiation, acting remodeling and invasiveness along with protection from apoptosis (Brinkmann and Schulz 2006).

2.1.6.6. Latent Membrane Protein-2 (LMP-2)

Latent Membrane Protein-2 gene encodes to LMP-2A and B which are transmembrane proteins that performance to block tyrosine kinase signaling LMP-2A and its splice variant LMP-2B that is not essential for EBV-induced cell transformation in B-lymphocytes, but aid in the transformation (Fukuda and longnecker 2007).

Migration and invasiveness potential of epithelial cells signals from LMP-2A mimic those of the BCR, which promote B lymphocyte survival and proliferation (Brinkmann and Schulz 2006).

Recent studies have pointed to an essential role of LMP-2A in promoting growth, transformation and survival in germinal center B cells which lost BCRs due to crippling mutation (Mancao and Hammerschmidt 2007).

2.1.6.7. Epstein-Barr Encoded Small RNA (EBERs)

The abundantly transcribed Epstein-Barr encoded small RNAs EBER-1 and -2 plays a role in System Lupus Erythematosus (SLE) and ribosomal protein L22. They also bind to the double stranded RNA-dependent protein kinase (PKR) and thereby, block the interferon alpha-dependent signal transduction pathway that EBERs have shown to exert anti-apoptotic and tumorigenic functions (Elia et al. 2004).

EBERs stimulate the expression of interleukin IL-10 in B-cells, insulin-like growth factor (IGF)-1 in gastric carcinoma and nasopharyngeal carcinoma cells, IL-9 in T cells that act as an autocrine growth factor. EBERs regulate apoptosis through diverse mechanisms (Nanbo et al. 2002).

Epstein-Barr virus encoded small RNA-2 has however a more prominent role in EBV mediated growth trasformation, as viruses lacking the coding sequence for this RNA were significantly less efficient in transforming lymphoplastoid cell lines (LCLs) *in vitro*. These observations have been extended to epithelial cells lines (Iwakiri et al. 2005).

2.1.6.8. Small Nucleolar RNAs (snoRNAs)

Generated by the virus during latency, these are a class of small RNA molecules that primarily guide chemical modifications of other RNAs, mainly ribosomal small nuclear RNAs. There are two chief classes of snoRNA, the C/D box snoRNAs, which are related to methylation and the H/ACA box snoRNAs, which are associated with Pseudouridylation. SnoRNAs are usually indicated to as leader RNAs but not be confused with the guide RNAs that direct RNA editing in trypanosomes (Bachellerie et al. 2002).

2.1.6.9. Epstein-Barr Virus-Stable Intronic Sequence RNA

It has two structured latent sisRNAs. EbvsisRNA-1 is predicted to interact with host miRNAs. Ebv-sisRNA-2 is modeled to have widespread and exceptionally thermodynamically stable RNA structure that is evolutionarily conserved in related herpes virus. Ebv-sisRNA-1 is generated during latency program III. After the EBERs it is the third most abundant small RNA produced by the virus during this program (Moss and Steitz 2013).

2.1.6.10. MicroRNA (miRNA)

A microRNA is containing about 22 nucleotids encoded by eukaryotic nuclear DNA in plants and animals and by viral DNA in certain viruses whose genome is based on DNA, miRNAs function via base pairing with complementary sequences within mRNA molecules.

MicroRNAs have two transcripts, one set near the BHRF1 cluster. The three BHRF1 miRNAs are expressed during type III latency, one set in the BART gene and large cluster of BART miRNAs are expressed during type II latency. The Bam HIA region transcripts (BARTs), also named complementary strand transcripts (CSTs) play a role in tumorgenesis (Buisson et al. 2009).

2.1.6.11. Epstein-Barr Virus -Viral Capsid Antigen (EBV-VCA)

The Epstein–Barr virus viral-capsid antigen (EBV-VCA) is the antigen targeted by anti-VCA antibodies. Like antibodies can be used in serology to diagnose infectious mononucleosis. In events with primary infection, the sensitivity of IgG (immunoglobulin) and IgM, anti-VCA testing has been estimated to be 100% reliable.

Finaly, there are some proteins like EBV-EA early antigen in lytic stage, EBV-MA membrane antigen and EBV-AN alkaline nuclease in lytic stage (Moss and Steitz 2013).

2.2. Infectious Mononucleosis (IM)

Also known as mono or glandular fever is an infection usually produced by the Epstein–Barr virus. Most people infected by the virus as children, when the disease produces little or no symptoms. In young adults, the disease repeatedly outcomes in fever, sore throat, enlarged lymph nodes in the neck and fatigue. Maximum people become healthier in two to four weeks; however, feeling tired may last for months. The liver or spleen may also become swollen. In less than one percent of cases splenic rupture may occur (Ebell et al. 2016).

2.2.1. Biological Background of Infectious Mononucleosis

In late nineteenth century, in 1885, the famous Russian pediatrician Nil Filatov reported an infectious procedure he called "idiopathic denitis" showing symptoms that corresponded to infectious mononucleosis and in 1889 a German balneologist and pediatrician, Emil Pfeiffer freely reported same cases that tended to cluster in families, for which he invented the term Drüsenfieber ("glandular fever") (Evans 2013).

2.2.2. Sign and Symptoms

2.2.2.1. Children

Before adolescence, the disease typically just produces flu-like signs, if any at all. When found symptoms tend to be like to common throat infections (mild pharyngitis)(Cohin et al. 2016).

2.2.2.2. Adolescents and Young Adult

Fever, sore throat, swollen mobile glands usually located around the back of the neck (posterior cervical lymph nodes) and sometimes throughout the body. Another major symptom is feeling tired. Headaches are common and abdominal pains with nausea or vomiting sometimes also occur, fatigue and a general malaise for months. Mild fever and body aches may also persist beyond 4 weeks (Bennett et al. 2014).

2.2.2.3. Older Adults

Infectious mononucleosis mainly affects younger adults. When older adults do catch the disease, they less often have characteristic signs and symptoms such as the sore throat and lymphadenopathy. They more likely have liver enlargement and jaundice. People over 40 years of age are more likely to develop serious illness (Odumade et al. 2011) (Figure 2.8).

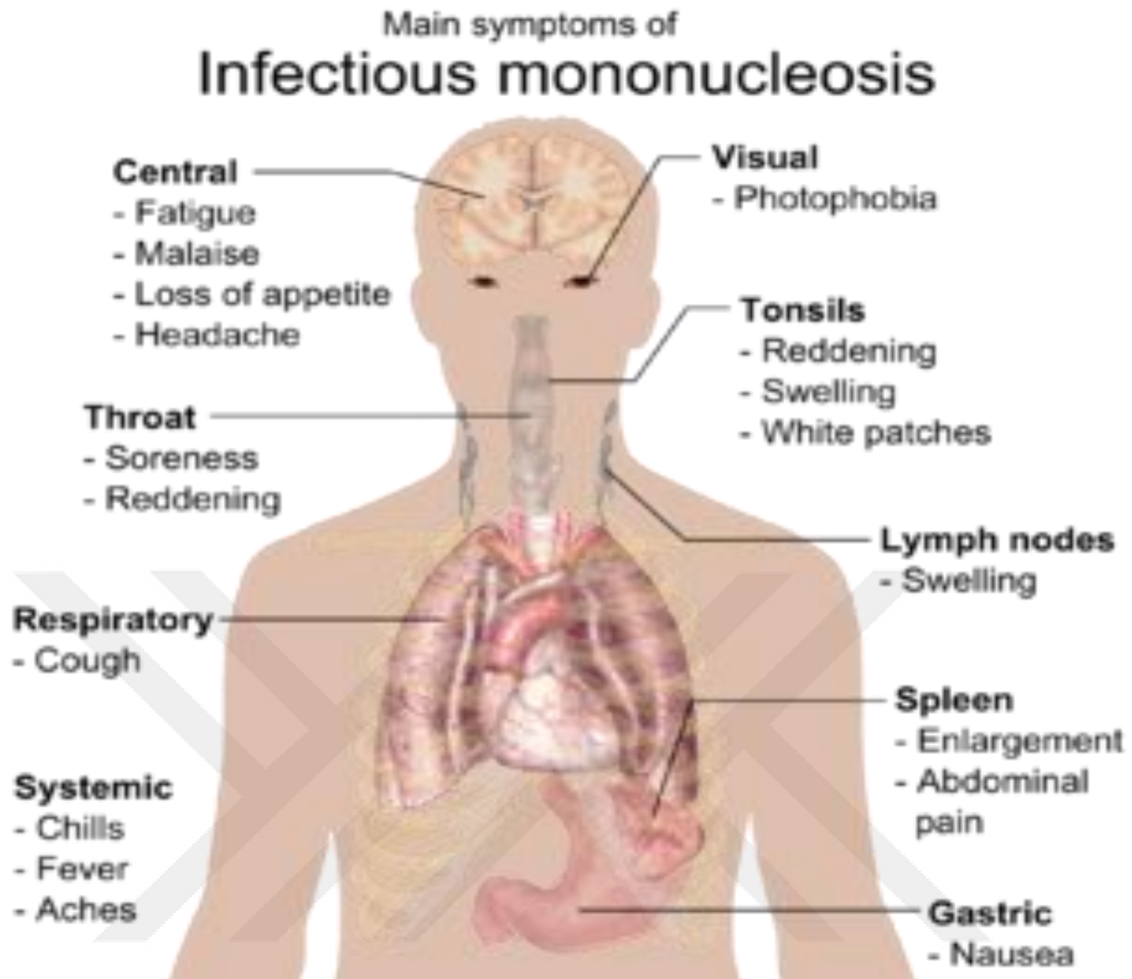

Figure 2.8. Main symptoms of infectious Mononucleosis (Shiel et al. 2013)

2.2.3. Incubation Period

The correct length of time between infection and symptoms is unclear. A review of the literature made an estimate of 33–49 days (Richardson et al. 2001).

In adolescents and young adults, signs are thought to appear around 4–6 weeks after early infection. Onset is often gradual and it can be rapid. The main symptoms may be preceded by 1–2 weeks of fatigue, feeling unwell and body aches (Cohen 2008).

2.2.4. The Main Causes for Infectious Mononucleosis

2.2.4.1. Epstein–Barr Virus

About 90% of cases of infectious mononucleosis (IM) have caused by the EBV. It is one of the most commonly found viruses throughout the world. About 95% of the population has been exposed to this virus at the age of 40, but only 15 to 20% of teenagers and about 40% of exposed adults actually become infected (Schonbeck and Frey 2011).

2.2.4.2. Cytomegalovirus (CMV)

A minority of cases of IM is caused by human cytomegalovirus (CMV), another type of herpes virus. It is found in body fluids counting saliva, urine, blood and tears. An individual becomes infected with it by direct interaction with infected body fluids. CMV is most usually transmitted through kissing and sexual intercourse. It can also be transferred from an infected mother to her unborn child. This virus is often silent because the infection is usually asymptomatic (Larsen 2009).

2.2.4.3. Transmission

Epstein–Barr virus infection is spread by saliva and has an incubation period of four-seven weeks. The chances of transmission to new individuls may be the highest during the first six weeks next infection. Some studies signify that an individual can spread the infection for many months, possibly up to a year and a half (Kohlhof 2009).

2.2.5. Pathophysiology

The virus replicates first within epithelial cells in the pharynx and later primarily within B cells. The host immune response involves cytotoxic (CD8-positive) T cells against infected B-lymphocytes, resulting in enlarged, atypical lymphocytes (Downey cells). Mononucleosis is sometimes accompanied by secondary cold agglutinin disease, an autoimmune disease in which irregular circulating antibodies are directed against red blood cells that can lead to a form of autoimmune hemolytic anemia (Ghosh et al. 2007).

2.2.6. Prognosis

Serious complications occur in less than 5% of cases:

1- CNS complications include meningitis, encephalitis, hemiplegia, Guillain–Barré syndrome, and transverse myelitis. Infection with the Epstein–Barr virus has also been proposed as multiple sclerosis (MS)(Ascherio et al. 2007).

2- Hematologic: Hemolytic anemia (Ghosh 2007).

3- Hepatitis with the Epstein–Barr virus is rare.

4- Upper airway obstruction from tonsillar hypertrophy is rare.

5- Fulminant disease course of immunocompromised patients is rare.

6- Splenic rupture is rare.

7- Myocarditis and pericarditis are rare.

8- Postural orthostatic tachycardia syndrome.

9- Chronic fatigue syndrome.

10- Cancers associated with the Epstein-Barr virus.

11- Mild jaundice (Pattle and Farrell 2006).

2.2.7. Prevention

The isolation of patients with IM is needless. Don't intimate kiss or share dishes, eating utensils and toothbrushes etc. with someone who has Mononeuclesis. It is spread via saliva from an infected individual into another one's mouth and also it transfer within donate blood (Etienne et al. 2013).

2.3. EBV-Associated Cancers

2.3.1. Burkitt's Lymphoma

Burkitt's lymphoma is a particularly aggressive lymphoma, the hallmark of which is a chromosomal translocation between chromosome 8 and either chromosomes 14, 2, or 22. Because of this translocation, the oncogene c-myc (chromosome 8) is juxtaposed to the immunoglobulin heavy-chain (chromosome 14) or light-chain genes (chromosomes 2 or 22) (Gulley 2001).

This aberrant configuration results in the deregulation of c-myc expression. The relationship between EBV, Burkitt's lymphoma, and the c-myc translocation is complicated by the existence of two types of Burkitt's lymphoma: endemic (EBV present) and nonendemic (EBV generally absent) (Manilov et al. 1986).

2.3.2. Hodgkin's Disease

Hodgkin's disease is characterized by an expansion of Reed-Sternberg cells, which are now postulated to be of B-cell lineage.

Several lines of evidence link EBV to Hodgkin's disease:

(1) A 4-fold increased risk in individuals with a past history of infectious mononucleosis.

(2) Increased anti- body titers to EBV viral capsid antigen.

(3) The detection of monoclonal EBV episomes. The role that EBV plays in Hodgkin's disease is still not fully understood. EBV gene expression follows the latency II pattern with EBNA-1, LMP-1, LMP-2A, LMP-2B and the EBERs being expressed (Herbst et al. 1993).

2.3.3. Non-Hodgkin's Lymphoma in Immune Defect

EBV is recognized primarily for its capacity to infect B cells, but it can also infect other cells. Several types of non-B-cell, non- Hodgkin's lymphoma are associated with EBV This review will focus on the two types in which EBV has been most directly inplicated:

nasal T/natural killer cell lymphoma and angioimmunoblastic lymphadenopathy (Weiss 2002). Nasal T/natural killer non-Hodgkin's lymphoma cells exhibit several unique cell antigens, expression of natural kiler cell marker CD56 and absence of T-cell receptor gene rearrangement (Davison et al. 1996).

2.3.4. Nasopharyngeal Carcinoma

Undifferentiated nasopharyngeal carcinoma is associated with EBV, whereas the association with the other two subtypes of nasopharyngeal cancer is controversial at best (Vasef et al. 1997).

Undifferentiated nasopharyngeal cancer affects mostly individuals in their mid-40s and is more common in men. Nearly every undifferentiated nasopharyngeal carcinoma is EBV positive, regardless of geographical origin (parkin et al. 2002).

In undifferentiated nasopharyngeal carcinoma, EBV infects the epithelial cells of the posterior nasopharynx in Rosenmuller's fossa in Waldeyer's ring. Although an EBV-compatible receptor on epithelial cells has not been found, a surface protein that is antigenically related to the B cell. CD21 receptor has been described and could conceivably be used as a point of entry by EBV. Alternatively, it has been suggested that EBV may gain entry into nasopharyngeal cells through IgA-mediated endocytosis (Lin et al. 1997).

2.3.5. Gastric Carcinoma

EBV presence varies from more then 90% in lymphoepithelioma like gastric carcinomas to between 5 and 25% in gastric adenocarcinomas. Whether EBV plays a pathogenic role in either of these two tumors is still unclear (Oda et al. 1993).

EBV may enter the gastric epithelium without the use of a receptor. It has been suggested that this is accomplished by the binding of IgA antibody with EBV particles derived from B-lymphocytes with the uptake of these particles by gastric epithelial cells (Fukuyama et al. 1994).

EBV may enter the gastric epithelial cells via a receptor other than the CD21 receptor. EBV exhibits a novel latency pattern in gastric adenocarcinoma that includes the production of BARF-1, a homologue to human colony exciting factor 1 receptor with intracellular adhesion molecule 1 and the absence of LMP-1 (Kume et al. 1999).

2.3.6. Breast Cancer

The association between EBV with breast cancer is controversial. Some studies have reported an EBV incidence in breast cancer tissue as high as 21–51%. Indirect support for an association of EBV with breast cancer comes from observations that:

(*1*) EBV is present in breast tissue, where it is detected in breast milk in some women.

(*2*) Transfection of EBV DNA stimulates growth of human breast milk cells.

(*3*) Some EBV-associated lymphomas occur in the breast.

(*4*) Breast cancer has epidemiological similarities to young adult Hodgkin's lymphoma.

EBV has been identified in benign breast tumors in immunosuppressed women. Breast epithelial cells can be infected by direct contact with EBV-bearing lymphoblastoid cell lines (Bonnet et al. 1999).

2.3.7. Leiomyosarcomas

Leiomyosarcomas are smooth muscle tumors. They are not associated with EBV in immune competent hosts but have been strongly correlated with viral infection in patients whose immune system is compromised by HIV or other factors. These observations also indicate that EBV is capable of infecting smooth muscle cells, a finding consistent with experimental evidence that the EBV receptor is present on those cells (McClain et al. 1995).

2.3.8. EBV-Associated Lymphomas in Immune Defect

There exist several distinct classes of EBV-associated lymphoproliferative disorders in immune compromised individuals. The most common gene expression pattern in these disorders is latency III.

2.3.8.1. X-Linked Lymphoproliferative Disorders

X-Linked lymphoproliferative disease is characterized by three major phenotypes: fatal or fulminant infectious mononucleosis, B-cell lymphomas and dysgammaglobulinemia. The gene responsible for this disorder has been mapped to the long arm of the X chromosome (Xq24) (Sayos et al. 1998).

This gene is important in T/B-cell homeostasis after viral infection. In particular, defects in this gene may lead to a decreased ability to control immune responses to viruses, including EBV (Howie et al. 2000).

2.3.8.2. Post-Transplant Lymphoproliferative Disorder (PTLD)

These heterogeneous lymphoproliferative disorder arise in the setting of therapeutic immunosuppression after organ transplantation (Cesarman and Mesri 1999). Nearly all forms of the disorder harbor EBV and these lymphomas tend to be aggressive. Their development is probably a multistep process. Iatrogenic immunosuppression leading to primary EBV infection or reactivation of latent EBV infection is followed by polyclonal expansion of B-cell populations with a selective growth advantage. These cells are susceptible to genetic changes and BCL-6 may be one of the first such genes altered (Knowles 1998).

The incidence of post transplant lymphoproliferative disorder ranges from 0.5 to 30% and varies greatly depending on the organ being transplanted, the EBV status of the transplant recipient with donor and the therapies used to achieve immunosuppression (Holmes and Sokol 2002).

2.3.9. AIDS-Related Lymphoproliferative Disorders

AIDS- related lymphoproliferative disorders are a heterogeneous group of illnesses that arise in the attendance of HIV-associated immunosuppression, a state that permits the unchecked proliferation of EBV infected lymphocytes. These aggressive disorders include both central nervous system and systemic lymphomas. Pleural effusion lymphomas also occur and often contain EBV in addition to human herpes virus 8.

AIDS-related central nervous system lymphomas are derived from germinal center B cells and almost always contain EBV (Cesarman and Mesri 1999).

The central nervous system lymphomas include immunoblastic and large non-cleaved lymphomas. The immunoblastic subtype expresses LMP-1 and BCL-2 but not BCL-6. The large no cleaved subtype express BCL-6 but not LMP-1 or BCL-2 (Larocca et al. 1998).

Since its discovery as the first human tumor virus, EBV has been implicated in the development of a wide range of cancers as cleared in the Table 2.2.

Table 2.2. Characterization of EBV-associated Malignancies (Gulley 2001): MC, mixed cellularity; IP-CNS, immunoblastic primary central nervous system lymphoma; LD, lymphocyte depleted; LP, lymphocyte predominant; NK, natural killer; NS, nodular sclerosing.

Malignancy	Subtype	EBV gene expression pattern	% EBV positivity
Burkitt's lymphoma	Endemic Nonendemic	Latency I	>95% 15–30%
Hodgkin's disease	MC LD NS LP	Latency II	70% >95% 10–40% <5%
Non-Hodgkin's lymphoma	Nasal T/NK Angioimmunoblastic Lymphadenopathy	Latency II Latency II	>90% Unknown
Nasopharyngeal carcinoma	Anaplastic	Latency II	>95%
Breast Cancer	Medullary carcinoma Adenocarcinoma	Not clear	0–51%
Gastric Cancer	Lymphoepithelioma-like Adenocarcinoma	Controversial novel LMP-1 negative Latency III	>90% 5–25%
Posttransplant lymphoproliferative disorders		Latency III	>90%
AIDS-associated lymphomas	IP-CNS Other	Latency III	>95% 30–50%
Leiomyosarcomas in immunosuppressed individuals	Leiomyosarcomas varies	Unclear	Frequent

2.4. Laboratory Tests

A. Blood Smear

The peripheral blood smear from patients with infectious mononucleosis demonstrates Downey cells, large lymphocytes with irregular ovoid, folded nucleus and abundant, moderately basophilic unregularly cytoplasm. The nuclear chromatin is coarse. Some had small indistinct nucleoli (Longmore et al. 2007) (Figure 2.9).

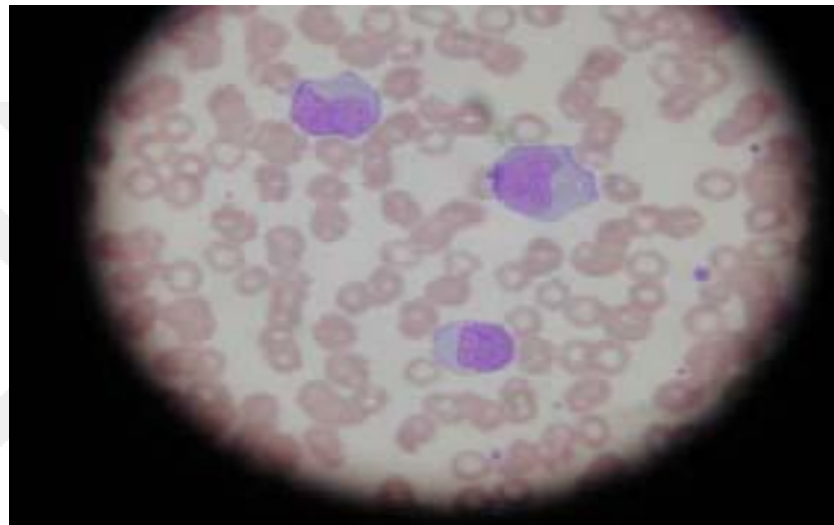

Figure 2.9. The peripheral blood smear (Uthman 2006)

B. Complete Blood Count (CBC)

A CBC is a panel of tests that evaluates the three types of cells that circulate in the blood.

a. Evaluation of white blood cells (WBC), the cells that are part of the body's defense system against infections and cancer and also play a role in allergies and inflammation. It identifies and counts the number of the various types of WBC present divided to two groups, Agranulocytes (Lymphocytes and monocytes) and Granulocytes (neutrophils, eosinophil and basophils).

b. Evaluation of red blood cells, the cells that transport oxygen throughout the body: Red blood cell (RBC) count is a count of the actual number of red blood cells in a person's sample of blood. Hemoglobin measures (HGB) mains the total amount of the oxygen-

carrying protein in the blood, (MCV) is a measurement of the average size of a single red blood cell. Mean corpuscular hemoglobin (MCH) is a calculation of the average amount of hemoglobin inside a single red blood cell. Mean corpuscular hemoglobin concentration (MCHC) is a calculation of the average concentration of hemoglobin inside a single red blood cell. Red cell distribution width (RDW) is a calculation of the variation in the size of RBCs. The CBC may also include reticulocyte count, which is a measurement of the absolute count or percentage of young red blood cells in blood.

c. Evaluation of platelets, cell fragments that are vital for normal blood clotting: The platelet count is the number of platelets in a person's sample of blood. Mean platelet volume (MPV) may be reported with a CBC. It is a calculation of the average size of platelets. Platelet distribution width (PDW) may also be reported with a CBC. It reflects how uniform platelets are in size. Patients with IM typically have an absolute lymphocytosis (>50% lymphocytes, with total lymphocytosis >5000/uL), and prominent atypical lymphocytes, so called Downey cells (> 10% of total leukocytes)(Napolitano et al. 2009).

C. Serological Tests

Serological tests for antibodies specific for Epstein-Barr virus (EBV) antigens are frequently used to define infection status and for the differential diagnosis of other pathogens responsible for mononucleosis syndrome. Using only three parameters [viral capsid antigen (VCA) IgG, VCA IgM and EBV nuclear antigen (EBNA)-1 IgG](Svahn et al. 1997).

1. Heterophile Antibodies:

The heterophile antibody is IgM that has differential absorption. It causes agglutination of sheep and horse erythrocytes but not guinea pig kidney antigen extract. The EBV heterophile antibody titers are highest during the first 4 weeks of disease. False negative reactions occur in 10% of adults and older children and often negative in children younger than 4 years of age (Svahn et al. 1997).

2. Specific EBV Antibodies

The specific tests for anti-EBV antibodies use different substrates or antigens and various technologies, of which those commonly used for the routine screening of EA IgG, EBNA-1 IgG, VCA IgG and IgM are:

1- Immunofluorescence assays (IFAs) usually use EBV-transformed B cell lines from patients with Burkitt's lymphoma.

2- Enzyme immunoassay (EIAs) use purified native or recombinant proteins, synthetic peptides or fusion proteins (complete proteins or fragments of the proteins encoded by the EBV genes).

3- Chemiluminescence immunoassay (CLIA) using synthetic peptides with different cut-off values for VCA IgM and EBNA-1 IgG can better distinguish the stage of infection and in the case of samples that are simultaneously EBNA-1 IgG, VCA IgG and IgM positive.

4- Enzyme-linked immunosorbent assays (ELISA) It is plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones.

5- Multiplex flow immunoassay (MFI) Antibodies to several antigen complexes may be measured. These antigens are the viral capsid antigen (VCA), the early antigen (EA) and the EBV nuclear antigen (EBNA). Testing for anti-VCA antibody is useful for identifying acute infection. Because serum antibody to EBNA is not present until several weeks to months after onset of infection, a positive anti-EBNA antibody excludes primary infection (Holmes and Sokol 2002) (Figure 2.10).

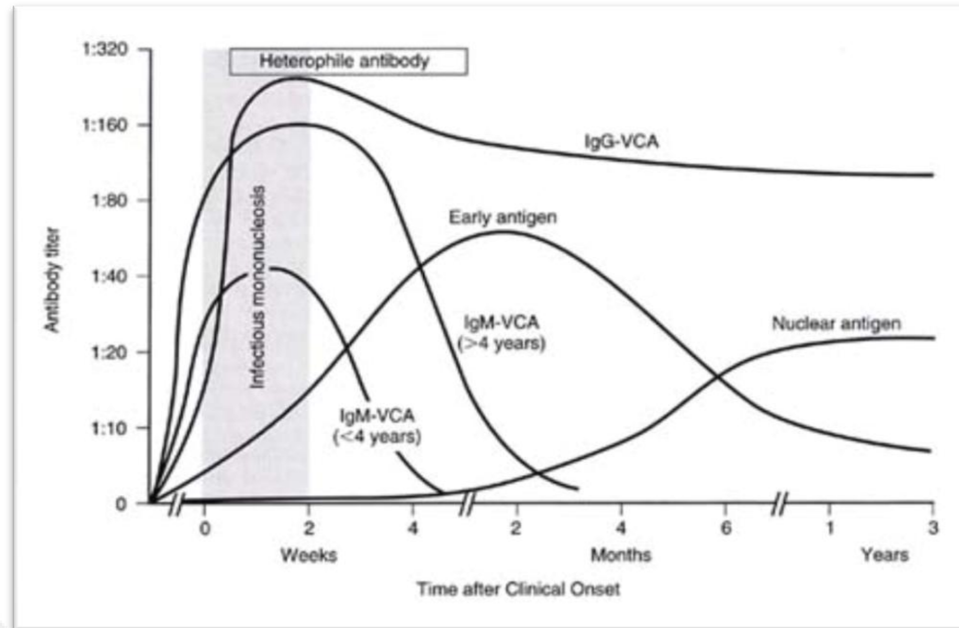

Figure 2.10. The antibodies to EBV in infectious Mononucleosis (Leath and Sumaya 2004)

3. Immunoblotting

In order to confirm the screening assays, various immunoblotting tests have been developed using viral lysates of EBV-transformed cells and recombinant antigens. It is considered that the latter is unaffected by antibodies against the cell material that can be found in patients with mononucleosis. Some line blots use recombinant antigens coated on the solid phase, such as EBNA-1, VCA, EA and MA (gp 350/250) (Schubert et al. 1998).

D. Diagnostic Tchnologies for Viral Associated Tumor

1. Electron Microscopy

Many investigations of the structure of virions or virus-infected cells involve electron microscopy. Large magnifications are achievable with a transmission electron microscope but the specimen, whether it is a suspension of virions or an ultra thin section of a viral infection cell must be treated so that details can be visualized. Negative staining techniques generate contrast by using heavy metal-containing compounds, such as potassium phosphotungstate and ammonium molybdate. In electron micrographs of virions the stains appear as dark areas around the virions (Carter et al 2007).

2. Immunohistochemistry (IHC)

Immunohistochemistry refers to the process of localizing of proteins in cells in a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). Immunohistochemistry is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue (O'Malle et al. 2006).

3. In Situ Hybridization (ISH)

The In situ hybridization (ISH) was first described in 1969 (Gall G.and Pardue M. 1969) and used radioactive labels for visualization. Although, this radioactive method reached a high level of sensitivity, it had practical drawbacks such as safety measures and the inability to distinguish more than one nucleic acid target simultaneously (Longnecher and Miller 1996).

In situ hybridization is usually applied to histological section or cell smears if performed carefully. In situ hybridization can provide not only the exact localization of target sequences but also excellent morphologic details of tissues or celluler contents and Epstein-Barr encoding region (EBER). In situ hybridization is the methodology of choice for the detection of the Epstein-Barr virus (EBV) in tissue sections (Unger et al. 2000).

4. Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) invented by Kary Mullis in 1983. It is an *in vitro* technique allows the amplification of specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequence.

When a sample is likely to contain a low number of copies of a virus nucleic acid, the probability of detection can be increased by amplifying virus DNA using PCR methods. PCR-based methods are used for strain determination (type-1 or 2) and EBV antigen (Newton and Graham 2003).

2.5. EBV Relation with the Bcl Family and Apoptosis

Ninteen members of the Bcl family have been characterized and divided into three subgroups:

(A) ‘Multi-domain’ anti-apoptotic members, containing multiple Bcl-2 Homology (BH) domains (BH1–4) promote cell survival. It includes Bcl-2-like long, Bcl-xL, Bcl-2, Bcl-2-like 2, Bcl-w, myeloid cell leukemia sequence 1 (MCL-1) and Bcl-2 fetal liver (bfl-1).

(B) ‘Multi-domain’ pro-apoptotic members, lacking just the BH4 domain characteristic of proteins that sponsor survival and induce cell death. It includes Bcl-2-associated X protein (Bax), Bcl-2-antagonist/killer (BAK), Bcl-2-associated agonist of cell death (Bad), and Bcl-2-related ovarian killer (Mtd/Bok).

(C) ‘BH3 domain-only’ members are sensors of distinctive cellular stresses that share sequence homology just in the BH3 domain. This subgroup contains the death-promoting Bcl-2 homolog (BIK), Bcl-2-interacting protein (Bid), *p53* up-regulated modulator of apoptosis (PUMA/Bbc3) and the Bcl-2-interacting mediator of cell death (BIM) (Kalla et al. 2010).

2.5.1. Impact of EBV Gene Products on Bcl-Family Members During Lytic Infection

2.5.1.1. BZLF1 Down-Regulates the Expression of Bcl-2 and Bcl-xL

The EBV immediate-early gene BZLF1 encodes the transcript factor Zta and is related to the cellular activating protein-1 (AP-1). In late primary infection, BZLF1 is expressed very early in B-cells. Its early expression does not immediately start the EBV lytic cycle but promotes the maintaining cell survival at the lytic phase. BZLF1might down-regulate the expression of the anti- apoptotic proteins Bcl-2 and Bcl-xL by down- controlling the major histocompatibility complex (MHC) class II-associated invariant chain (CD74) in CD4+ T cells (Lantner et al. 2007).

2.5.1.2. Apoptosis Regulator BHRF1

BHRF1, as a latent and lytic gene, comprises three conserved BH domains, BH1–3, which are characteristic of the Bcl-2 family. BHRF1 and Bcl-2 have a similar cellular distribution, both primarily localized in the mitochondrial endoplasmic reticulum and nuclear membranes. The anti-apoptotic activity of BHRF1 is not exactly equal to that of Bcl-2. The activities of BHRF1 and Bcl-2 are modulated by exclusive mechanisms. BHRF1 may use its pro-survival function in a manner similar to that previously found for human anti-apoptosis Bcl-2 members (Hickish et al. 1994).

A. BHRF1 and Bok

A pro-apoptotic member of the Bcl-2 family, Bok, has a high expression of messenger RNA (mRNA) in exclusive tissues such as the testis, ovary and uterus. Bok interrelates only with EBV BHRF1, MCL-1and bfl-1 and as a direct protein-protein interaction assay in vitro. In a variety of cell types, Bok induces cell killing, but this is inhibited following co-expression with BHRF1 and MCL-1. These findings suggest that Bok plays a sole role in apoptosis and further indicate that Bok-expression may be a target for the EBV-encoding anti-apoptotic protein BHRF1 (Yin et al. 1997).

B. BHRF1 Directly Counters BAK

BAK is an oligomeric mitochondrial membrane protein whitch plays an essential function when the mitochondrial release of apoptogenic factors initiates apoptosis. In a lymphocyte cell line, EBV BHRF1 binds BAK and the cell dies when it receives signals from the messenger BIM (Desbien et al. 2009).

BHRF1 changes its structure to accommodate the BAK domain and therefore keeps the BAK inactive. BHRF1 does replace BAK in cell. It has been shown to repress the activation of Bax and BAK to preserve mitochondrial function (Kvansakul et al. 2010).

C. BHRF1 and BOD

The Bcl-2-related ovarian death gene (BOD) was primary recognized as an ovarian Bcl-2-related, Bcl-2 homology (BH3) domain-only protein in an ovarian fusion cDNA library.

It has three variants (short, medium and long), all of which comprise a consensus BH3 domain without the other BH domains detected in channel-forming Bcl-2 family members (Hsu et al. 1998).

D. BHRF1 Interacts with BIK, Bcl-2 and Bcl-xL

BIK, a strong pro-apoptotic protein, shares just the BH3 domain and the C-terminal trans-membrane domains. BIK interrelates with the viral anti-apoptotic protein EBV BHRF1, and various cellular anti-apoptotic proteins, such as Bcl-2 and Bcl-xL and that this activity of BIK is inhibited following co-expression of EBV-BHRF1, Bcl-2 and Bcl-xL (Boyd et al. 1995).

E. BHRF1 Binds to BIM

BIM exists in three major isoforms and is expressed in a diversity of cell types, especially lymphocytes.It is a key regulator of life and death decisions (Anderton et al 2008). EBV BHRF1 binds to the BH3-only peptide BIM and interacts strongly with its protein and BHRF1 blocks apoptosis by binding to a crucial, lethal fraction of the BIM. The structure of BHRF1 in complex with BIM confirms that BHRF1 can counteract BIM directly (Kvansakul et al. 2010).

F. BHRF1 inhibits BH3-only proteins PUMA and Bid

PUMA and Bid are both BH3-only proteins and are required for the induction of apoptosis. BHRF1 is associated with PUMA and Bid. EBV BHRF1 promotes cell survival by directly repressing pro-apoptotic Bcl-2 family proteins counting PUMA with Bid (Kvansakul et al. 2010).

The structure of BHRF1 in complex with PUMA or Bid is still unclear. So, it is possible that BHRF1 changes its structure to accommodate the PUMA or Bid domain, because Bid is measured to be an activator like BIM (Letai et al. 2002).

2.5.1.3. Apoptosis Regulator BALF1 Associates with Bax and BAK

EBV BALF1 fails to protect cells from Bax-induced apoptosis in the DG75 B cell, so it lacks an anti-apoptotic function. The function of BALF1 is not yet clear, but it remains possible that BALF1 interrupts the apoptotic pathway in EBV-infected cells by interaction with pro-apoptotic proteins, such as Bax and BAK (Bellows et al. 2002).

2.5.1.4. Bam HI-A Region Fragment1 Activates Bcl-2

The EBV Bam HI-A rightward frame 1 (BARF1) gene is interpreted to procedure an early protein that is homologous to the intercellular adhesion molecule 1 (ICAM-1) and the human cloning stimulating factor I receptor (HCSF-IR). BARF1 activity includes both an immunomodulatory function and oncogenicity. BARF1 protein modulation of the host immune response to the virus is achieved by the protein acting as a receptor of human colony-stimulating factor (Hcsf-1) and as an inhibitor of α-interferon secretion from mononuclear cells (Sheng et al. 2001).

2.5.2. Impact of EBV Gene Products on Bcl-Family Members During Latency

2.5.2.1. EBNA2 Interacts with Various Bcl Family Genes

EBV nuclear antigen 2 (EBNA2) is the first latent-cycle protein of EBV and is essential for B-cell immortalization, proliferation and survival. EBNA2 up-regulates most other anti-apoptotic proteins like bfl-1, Bcl-xL, Bcl-2 and MCL-1 and induces expression of the pro-apoptotic proteins BIM and Bid in EBNA2-expressing cells (Kohlhof et al. 2009).

2.5.2.2. EBNA3A and EBNA3C Repress BIM Expression

EBNA3A and EBNA3C are responsible for effective immortalization in EBV- infected B cells. The inhibition seems to be predominantly directed at the regulation of BIM mRNA levels. EBNA3A and EBNA3C together inhibit the initiation of BIM transcripts. EBNA3C is directly targeted to the BIM promoter (Paschos et al. 2012).

2.5.2.3. Latent Membrane Protien-1

A. LMP-1 Specifically up-Regulates Bcl-2 Expression

LMP-1 up-regulates adhesion molecules for example cell adhesion molecule 1 (CAM-1), by activating NF-κB, leading to the up-regulation of Bcl-2 expression in B-cell lines (Rowe et al. 1994).

The intracellular carboxyl-terminal cytoplasmic region of LMP-1 has been shown to intract with signaling molecules involved in the tumor necrosis factor receptor (TNFR) family-activated pathway, including the transmembrane protein CD40. Then activates the NF-κB/C-jun N-terminal kinase/AP-1 pathway and finally regulates Bcl-2 expression (Noguchi et al. 2001).

B. LMP-1 Mediates MCL-1 Expression

MCL-1 was first recognized as a novel EBV gene active in early cell variation induced in a human myeloid leukemia cell line. MCL-1 protects Chinese Hamster Ovary cells from apoptosis caused by c-MYC over-expression and it erodimerizes with Bax. Because in EBV-infected B cells the regulation of MCL-1 by LMP-1 occurs at an early stage, prior to up-regulation of Bcl-2 and decreased MCL-1 levels, it is optional that MCL-1 functions as a rapid, crucial immediate-early response effector of cell survival (Kozopas et al. 1993).

C. LMP-1 Drives the Anti-Apoptotic bfl-1 Gene

B-lymphocyte decision depends on activation of the NF-κB signaling pathway and the CD40 receptor and involves the participation of (TNFR)-associated factor 2. It is mediated through the carboxyl terminal activate region 2 (CTAR2) associated with the CTAR1 region of LMP-1 (Kim et al. 2012).

2.5.2.4. LMP2A Increases the Expression of Bcl-xL and Bcl-2

LMP2A activates H-Ras and in turn preferentially activates the phosphatidyl-inositol 3-kinase (PI3K)/Akt pathway, normally imparting an essential survival signal in response to B-cell receptor signaling (Wang et al. 1996).

2.5.2.5. EBER-1 and EBER-2 up-Regulate Bcl-2 Protein Expression

EBV inhibition of apoptosis and up-regulation of the Bcl-2 protein are important for the malignant phenotype. Previous reports also provided straight evidence that EBV up-regulates Bcl-2 expression by repressing the activation of the double-stranded RNA-dependent protein kinase (PKR). This allows c-MYC to exert its oncogenic function and eventually results in the inhibition of apoptosis (Wong et al. 2005).

2.5.2.6. MIR-BART5 Suppresses PUMA Expression

MIR-BART5 promotes host cell by targeting PUMA expression and contributes to the establishment of latent infection in nasopharyngeal carcinoma and EBV germinal center cells (Zhang et al. 2010).

EBV infection disrupts normal cell death and survival pathways in various human tumor cells, including lymphocytes, fibroblasts, and epithelial cells. Interconnected signaling pathways mediate apoptosis. Pro-apoptotic signals are mediated by BZLF1, down-regulating Bcl-2 and Bcl-xL expression involving the repression of CD74 and p65. Pro-survival pathways occur mainly via EBV gene products (shown in light green).

The activation of BHRF1 results in repression of the pro-apoptotic proteins BIM, PUMA, and Bid; the activation of BARF1 causes the up-regulation of pro-survival Bcl-2 protein levels; LMP-1 stimulation results in the CTAR1 and CTAR2 regions of LMP-1 interacting with TNFR/CD40, including TARF1, and then activates the NF-κB/c-JNK/AP-1 pathway and CAM-1, and finally up-regulates bfl-1 and Bcl-2 expression.

In addition, the activation of LMP-1 inhibits or activates MCL-1; MIR-BART5 stimulation results in the repression of the pro-apoptotic protein PUMA; the activation of LMP2A results in activation of the Ha-Ras, PI3K/Akt pathway and the Raf/MEK/ERK pathway as well as NF-κB, and then increases the expression of Bcl-xL and Bcl-2

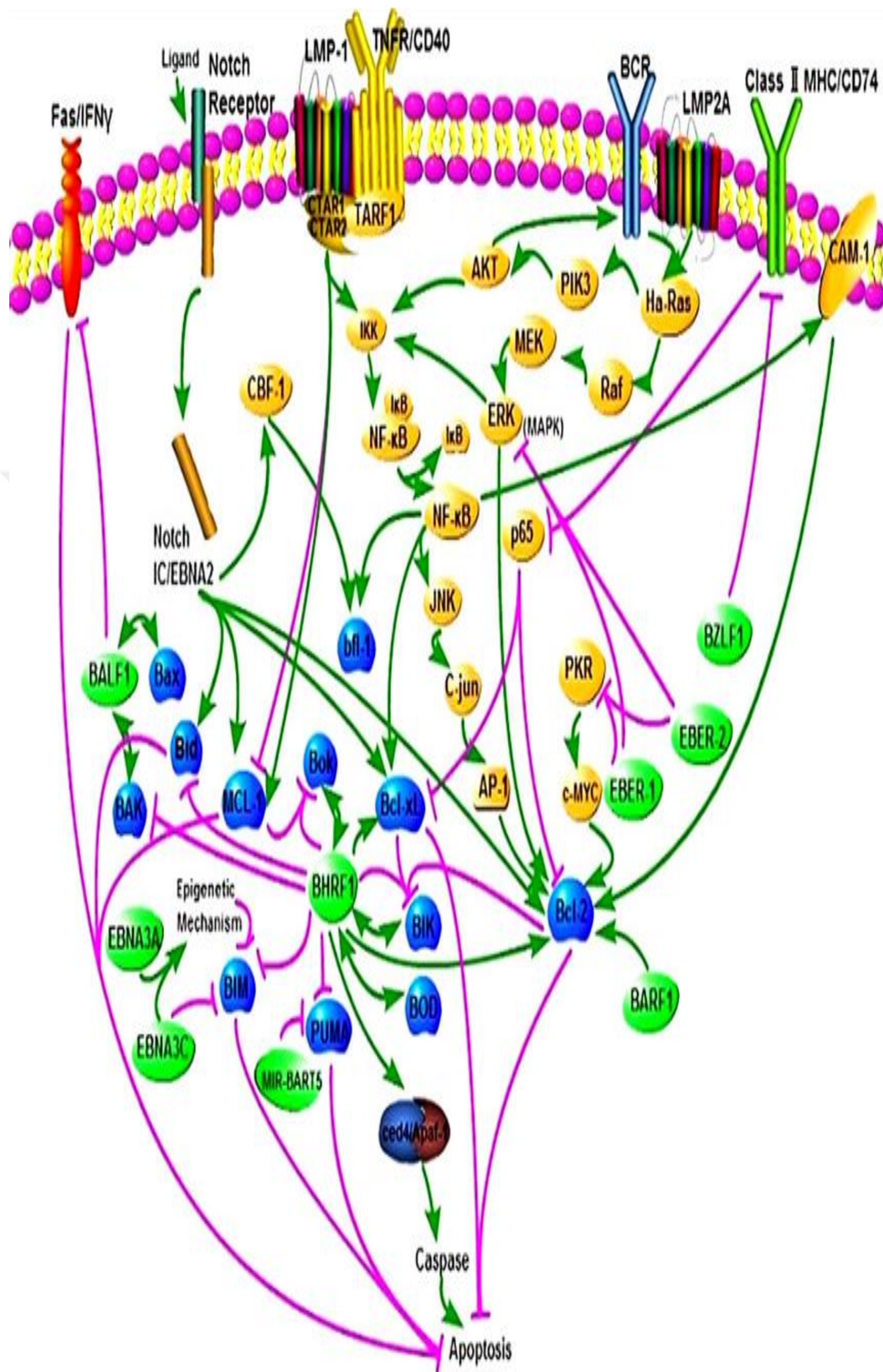

Figure 2.11. Epstein-Barr Virus interactions with the Bcl-2 protein family and Apoptosis (Qin and Zherg 2013)

All of these result eventually in the inhibition of apoptosis. BHRF1 is known to interact with the pro-apoptotic proteins Bok, BAK, BOD and BIK. BALF1 interacts with Bax and BAK. Likewise, EBNA2 interacts with the prosurvival proteins Bcl2L1, Bcl-2, and MCL-1 and the pro-apoptotic proteins BIM and Bid. EBNA3A and EBENA3C inhibit BIM expression, and EBNA3C targets BIM directly. EBER-1 and EBER-2 up-regulate Bcl-2 expression by inhibiting PKR or MAPK and C-jun. EBNA2, mimicking Notch-IC, activates CBF-1 to up-regulate bfl-1.

EBV also impacts the apoptosis pathway via the FAS/IFN-γ receptor and by binding to ced4/Apaf-1. Interestingly, the Bcl-2 family members, with which diverse EBV gene products interact, are essential in the 'decision' step of apoptosis, and then the activation of caspases mediates the 'execution' phase. These are both important steps in apoptosis.

2.6. The Immune System

Immune system is designed to fight against different pathogens. Many viruses infect the immune cells for the propagation of new progenies, thus the infection may modulate the host immune homeostasis. EBV is infecting B cells and impact host immune system more broadly than previously thought, for example the development of regulatory NKT subsets (Mei 2011).

Primary immunodeficiency diseases occur in persons born with the immune system that is either absent or in a weak position in its ability to function People with Primary immunodeficiency cannot get rid of germs or defend themselves from germs and causing frequent infections and other problems that are hard to cure, infections may appear to be common illnesses such as sinus and ear infections, pneumonia, fever, common colds and bronchitis (Lim and Elenitoba 2004).

The immune system defends the body from infection throuth a series of complex interactions involving antibodies, plasma cells, sensitized T- and B-cells, circulating complemant proteins, soluble, soluble mediators (cytokines), neutrophils, macrophages and dozens of other mediator and celluler components complicated in immunoregulation. Defects in any part of an immune response can impair the body's ability to protect itself against invasion in an increasing chance of severe infection (Lim and Elenitoba 2004).

2.6.1. Immune Evasion by Epstein-Barr Virus

EBV starts a latent infection in B-lymphocytes characterized by controlled viral gene expression. For the production of new viral progeny, EBV reactivates from these latently infected cells. Through the productive phase of infection, a range of over 80 EBV gene products is expressed, presenting a vast number of viral antigens to the primed immune system. In particular the EBV-specific CD4+ and CD8+ memory T lymphocytes are able to respond within hours, possibly destroying the virus-producing cells before viral replication is completed and viral particles have been released.

Preceding the adaptive immune response, potent innate immune mechanisms provide a first defense in the primary and recurrent infections. In spite of this broad range of antiviral immune effector mechanisms, EBV persists for life and continues to replicate because EBV has evasion strategies. These evasion mechanisms may also compromise the elimination of EBV-transformed cells and thus contribute to malignancies associated with EBV infection (Ning 2011).

2.6.2. EBV Infection in the Immune Competent Host

EBV persists in the B-lymphoid system and has the ability to drive B-cell growth through coordinated expression of a unique set of latent cycle genes. Little is known about these very early events. Lytic replication in oropharyngeal epithelium and possibly also locally infiltrating B cells are occurs too.

The virus appears to spread into the B-cells by activation of a latent growth-transforming infection. More of these expanding cells express the full panel of virus latent proteins and, by implication, resemble the lymphoblastoid cell lines (LCLs) that arise when the virus transforms normal B-cells in vitro. The growth-transforming program is suppressed and those cells, now carrying the virus genome as a truly latent (antigen-negative) infection, enter the recirculating memory B cell pool (Juszczynski et al. 2007).

2.6.3. Biological Function of T-Cells and NK Cells

The immune response is clearly critical for the control of EBV from primary immunodeficiencies that affect natural killer cells (NK) or T lymphocytes (Hislop et al. 2005).

Early-differentiated NK cells might play a key role in the immune control of primary infection with this persistent tumor-associated virus. NK cells are characterized by the absence of T-cell receptor (TCR) gene reorganization, absence of expression of the TCR-CD3 complex and the expression of CD16 and CD56. NK cells and cytotoxic T cells share a close relationship in terms of ontogeny and function (Rickinson 2014).

The T-cell compartment is split into $CD4^+$ plus $CD8^+$ T cells; these are mentioned to T-helper and cytotoxic T cells, respectively. These cells play critical roles in the immune system and in the regulation of immune responses (Lanier et al. 1992).

The immune-modulating mechanisms evolved by the universal but possibly oncogenic Epstein–Barr virus with the molecular mechanisms of genes interfering with HLA class I antigen presentation or virus evasion (Delecluse et al. 2007).

Acute infectious mononucleosis is characterized by abnormally high numbers of circulating CD8+ T cells. The T-cell response is directed at both lytic and latent infections in carriers. T lymphocytes directed against EBV-infected B cells. During convalescence, CD8+ T cells return to normal levels and antibodies develop against EBV nuclear antigen-1 (Henry et al. 2015).

2.6.4. CD56 bright CD16 Cells and CD56 dimCD16 Cells

Human NK cells are divided into CD56bright CD16 cells besides CD56dim CD16 cellsCD56bright cells can differentiate into CD56dim both in vitro. This differentiation was inhibited by fibroblast growth factor receptor-1 Ab, demonstrating a role of the CD56. CD56bright NK cells had longer telomere length compared with CD56dim NK cells, implying the former are less mature (Balfour et al. 2013).

Human NK cells are classified into two populations according to the intensity of CD56 (neural cell adhesion molecule (NCAM2) surface expression, as well as possession of CD16, the FcγRIII. CD56dimCD16bright make up nearly 90% of circulating NK cells and play a key role in natural and Ab-mediated cell cytotoxicity. CD56brightCD16/dim comprises the remaining 10%CD56bright NK cells predominate in lymph nodes and sites of inflammation. Their cells produce abundant cytokines (e.g., IFN-γ) and have immunoregulatory function (Antoni et al. 2007).

CD56 bright NK cells link adaptive and innate immunity and express the HA IL-2R and IA IL-2R and also have a distinct NK receptor receptor repertoire with expression of the C-type lectin CD94/NKG2 family and little expression of killer cell Ig-like receptors (KIR) (Antoni et al. 2007).

3. MATERIALS AND METHODS

3.1. Subjects and Specimens

This study was carried out during the period beginning from February 2015. The pathological samples were collected during the period from May 2016 to the end of August 2016. Three ml of venous blood was collected in EDTA tube to each of the thirty samples. The blood samples were collected from Hiwa Hospital cancer pateints in Sulymaniyah city.

3.1.1. Patients Study Groups

Thirty blood samples were collected from cancer pateints and divided to three groups; Nasopharyngeal Carcinoma, Gastric Carcinoma and Breast cancer. The samples were collected from female and male patients of ages from 25 to 55 years.

3.1.2. Healthy Control Group

Blood samples were collected from ten control healthy individuals that did not have signs of cancer. These control samples were collected from females and males of ages from 25 to 50 years.

3.2. Standard PCR

3.2.1. Materials and Instruments

3.2.1.1. Equipment

Table 3.1. List of Equipment

No.	Name	Company	Country
1	Refrigerated Microcentrifuge	Haier	Germany
2	Microcentrifuge	SIGMA 1-15	Germany
3	Thermocycler	TECHNE TC-312	U.K.
4	Vortex	Vortex 2Gene G-560E	USA
5	Sensitive balance	BOSCH SAE 80/200	Germany
6	Electrophoresis power Supply	BIOTEC-FISCHER Consort EV231	Germany
7	Gel Electrophoresis Chamber	BIOTEC-FISCHER PHERC-sub 1010-E	Germany
8	Microwave	Whirlpool AVM585	Philippines
9	Water bath	GFL 1083	Germany
10	Freezer -20°C	VESTEL	Turkey
11	Ice maker	FIOCCHETTI	Italy
12	Autoclave	Systec	Germany
13	Incubator	memmert	Germany
14	Refrigerator	VESTEL	Turkey
15	Microscope	Olympus	Japan
16	Rack	Biolab	Taiwan
17	UV light	Olympus	Japan

3.2.1.2. DNA Primer

Table 3.2. List of DNA Primer

No.	Primers	Amplicon	Company	Country
1	F-CCCTAGTGGTTTCGGACACA R-ACTTGCAAATGCTCTAGGCG	108-bp	metabion	Germany
2	F-AAATTTTAACCATGAGGGAAATC R-GTGGGGTCGATGCCATGT	104-bp	metabion	Germany

3.2.1.3. Kits

A. Taq DNA polymerase Kit QIAGEN (Cataloc No.201223)

B. QIAamp DNA Mini and Blood Mini Handbook
For DNA purification from whole blood (QIAamp DNA Mini Kit) Cataloc no: 51304

3.2.1.4. Ethidium Bromide

It is prepared by manufacturer and was ready to usage.

3.2.1.5. DNA Ladder

It is prepared by manufacturer and was ready to usage.

3.2.1.6. DNA Loading Dye

DNA Loading Dye is prepared by manufacturer and was ready to usage.

3.2.2. Methods

3.2.2.1. Sample Collection

Three ml of venous blood was collected in EDTA tube each of the thirty samples (from beginning of May 2016 to the end of August 2016). Blood sample were transported in cool box to laboratory and tested after 3 hours from taking the samples.
Blood samples were collected from ten persons that did not have signs of cancer and considered as control.

3.2.2.2. Protocol: DNA Purification from Blood (Spin Protocol)

The principle of DNA purification depends on operating high salt concentration. The protocol is for purification of total DNA from whole blood, we should know that:

1-All centrifugation stages are carried out at room temperature (15-25°C).

2-200µl of whole blood yields 3-12µg of DNA.

3- Heat a water bath to 56°for using in step four. If a precipitate has formed in buffer AL dissolved by incubating at 56°C.

3.2.2.3. Procedure

1- Pipet 20µl QIAGEN Protease (proteinase K) into the end of a 1.5 ml micro centrifuge tube.

2- Add 200µl of blood sample to the microcentrifuge tube.

3- Add 200µl of Buffer AL to sample and mix it by puls- vortexing for 15 seconds.

4- Incubate at 56°C for 10 minutes.

5- Kindly centrifuge the 1.5ml micro centrifuge tube to eliminate drops from inside the lid.

6- Add 200µl ethanol 96% to the sample and mix it again by vortex for 15seconds. Then, kindly centrifuge the 1.5ml microcentrifuge tube to eliminate drops from inside the lid.

7. Wisely apply the mixture from step 6 to the QIAamp Mini spin column (in a 2ml collection tube).

Close the top and centrifuge at 6000xg (8000rpm) for 1 minute, put the QIAamp Mini spin column in a clean 2ml collection tube and discard tube that has the filtrate.

8. Wisely open the QIAamp Mini spin column and add 500µl Buffer AW1 without weting the edge. Close the top, and centrifuge at 6000xg (8000rpm) for 1 minute, put the QIAamp Mini spin column in a clean 2ml collection tube and discard the tube that has the filtrate.

9- Wisely open the QIAamp Mini spin column and add 500µl Buffer AW2. Close the top and centrifuge at high speed (20,000x g; 14,000rpm) for 3minutes.

10- Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the ancient collection tube with the filtrate. Centrifuge at high speed for 1minute.

11-Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube that has the filtrate. Wisely open the QIAamp Mini spin

column and put 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 minute and the centrifuge at 6000x g (8000rpm) for 1 minute. Finally, put the final result in the PCR tube for working on PCR or store it at -20°C until collection all the samples.

3.2.2.4. PCR Amplification and Agarose Gel Electrophoresis

A. PCR Amplification and Agarose Gel Analysis with Internal Control

The DNA region from EBER gene was amplified by PCR using specific oligonucleotide primers. All 30 patient samples and 10 control samples were tested for the mutation using 1 µl 10µM of F- EBER gene (0.4µM), 1 µl 10µM of the R-EBER gene (0.4µM), 1 µl 10 µM of F- beta-tubulin gene (0.4µM), 1 µl 10µM of the R-beta-tubulin gene (0.4µM), 3 µl of 10X PCR buffer, 1 µl of dNTP, 13 µl of D.W., 0.5 µl of Tag polymerase and 4µl of DNA sample were used in a final volume of 25 µl.

Amplification includes initial denaturation for 4 minutes at 94°C, then 30 cycles: each cycle consists of Denaturation at 94°C for 30 seconds. Annealing at 59°C for 30 secconds, Extension at 72°C for 30 seconds (according to primer manufacturer) and final extension 72°C for 5 minutes.

The amplification products were analyzed on 2-3% of agarose gel at 90 V, 100 mA, 9 W for 60 minutes.

B. PCR Amplification and Gel Analysis without Internal Control

The DNA region from EBER gene was amplified by PCR using specific oligonucleotide primers. All 30 patient samples and 10 control samples were tested for the mutation using 1 µl 10µM of F-EBER gene (0.4µM), 1 µl 10µM of R-EBER gene (0.4µM) 1 µl 10µM of the R-beta-tubulin gene (0.4µM), 3 µl of 10X PCR buffer, 1 µl of dNTP, 15 µl of D.W., 0.5 µl of Tag polymerase and 4µl of DNA sample were used in a final volume of 25 µl.

Amplification include initial denaturation for 4 min. at 94°C, then 30 cycles: each cycle consists of Denaturation at 94°C for 30 seconds. Annealing at 59°C for 30 seconds, Extension at 72°C for 30 seconds (according to primer manufacturer) and final extension 72°C for 5 minutes.

The amplification products were analyzed on 2-3% of agarose gel at 90 V, 100 mA, 9 W for 60 minutes.

3.3. Real- Time PCR (RT-PCR)

DNA extraction kit was also used in this method.

3.3.1. Principle of the Procedure

The principle in Real-time PCR the amplified product is monitored via fluorescent dyes. These are typically linked to oligonucleotide probes and bind specifically to the amplified product.

Monitoring the fluorescence light forces during real-time PCR allows detection and quantitation of the accumulating product without the need to open the reaction tubes after the PCR run.

3.3.2. Prelimary Preparing

1. Make sure that at least one quantitation standard as well as one negative control (Water, PCR grade) are included per PCR run.

2. To generate a standard curve, use all 4 quantitation standards provided (EBV RG QS 1-4) for each PCR run.

3. Make sure that the cooling block (accessory of the Rotor-Gene Q Instrument) is precooled to 2-8°C.

4. All reagents need to be thawed completely and mixed by quick vortex and centrifuged briefly.

3.3.3. Procedure

1. Place the desired number of PCR tube into the adapters of the cooling block.

2. If used the internal control to monitor the DNA isolation procedure and to check for possible PCR inhibition, follow step A. If used the internal control exclusively to check for PCR inhibition, fellow step B.

A. The internal control has already been added to the isolation.

B.The internal control must be added directly to the mixture of EBV RG Master. Internal control used alone to check PCR inhibition.

3. Pipet 30 µl of the master mix into each one of PCR tube and add 20µl of the eluted sample DNA. Similarly, 20µl of at least one of the quantitation standards (EBV RG QS 1-4) must be added as a positive control and 20µl of (water, PCR grade) as a negative control.

4. Close the PCR tubes. Make sure that the locking ring (accessory of the Rotor-Gene Instrument) is placed on top of the rotor to prevent accidental opening of the tube during the run.

5. For the detection of EBV DNA, create a temperature profile according to the following steps.

a- Setting the general assay parameters.

b- Initial activation of the hot-start enzyme.

c- Amplification of the DNA.

d- Adjusting the fluorescence channel sensitivity.

e- Starting the run.

6. First, open the New Run Wizard dialog box. Check the locking Ring Attached box and tick next.

7. Select the PCR reaction volume and tick next.

8. Tick Edit Profile button in the next New Run Wizard dialog box, and program the temperature profile.

9. The detection rate of the fluorescence channels has to be determined depending on the fluorescence intensities in the PCR tubes.

Tick Gain Optimisation Setup dialog box. Set the calibration temperature to 65°C to match the annealing temperature of the amplification program.

10. The development values determined by the channel calibration are saved automatically and are listed in the last menu window of the programming procedure. Teck Start Run.

3.3.4. Quantitation

The enclosed quantitation standards (EBV RG QS 1-4) are used (as earlier purified samples) and the same volume is used (20µl).

To make a standard curve on Rotor-Gene Q Instruments, all 4 quantitation standards should be used and defined as standards with the specified concentration.

The quantitation standards are defined as copies/µl. The following calculation has to be applied to convert the values determined using the standard curve into copies/ml of sample material:

$$\text{Result (copies/ml} = \frac{\text{Result (copies/}\mu\text{l)} \times \text{Elution Volume (}\mu\text{l)}}{\text{Sample Volume (ml)}} \qquad (3.1)$$

The results in the final shown the the pesron's that has the virus or not throuth the table

3.4. Immunoblot Method

3.4.1. Materials and Instruments

1- SDS-PAGE gel (Separating gel and Stacking gel).

2- Buffers (PBS buffer, RIPA buffer, Running buffer,Transfer buffer,TBS-T buffer, sample buffer and Laemmli buffer).

3- Sandwich cassette (Nitrocellulose paper,Wet fibre pad and Fiber paper).

4- Antibodies (1-Anti-CD56/NCAM-1) and (2-Anti-Rab. HRP N2013).

5- Detection solution (Luminal solution and Hydrogen Beroxide solution).

6- Tools (Forceps, clean plastic test tube, gloves, razor blade, Parfilm tip, Micropipete (different size),Tapmicropipete (different size), Palp pipette and Solution tube).

7- Glassware (Conical flask 1000ml,Becker 500ml,Graduated cylinder 50 ml).

8- Power supply.

3.4.2. Sample Preparation

3.4.2.1. Blood Collection

Three ml of whole blood was collected from patients and control indivitules and was added directly into EDTE tubes with gently mix.

EDTA tubes were transported in a cool box to laboratory and used after three hours from sample collection.

3.4.2.2. White Blood Cell Isolation

1- Centrifuge the EDTA tube that has three ml of whole blood at 2.500Xg for 5 minutes. Discard the serum and plasma.

2- Wash the rest in PBS buffer (Phosphate Buffered Saline), the ratio of PBS buffer to blood cells is (3:1), by centrifugation at 2.500Xg for 20 minutes.

3- The buffy coat absorped from the middle membrane be special peppete.

4- Add PBS buffer, the rate is (18:1) Buffy coat to discard the platelet.

5- Add cold RIPA buffer (Radioimmunoprecipitation assay) to the cell pellet, use 1000ml of RIPA buffer for 100ml of leukocytes.

6- Pipette the mixture up and down to suspend the pellet.

7- Shake mixture gently and centrifuge mixture at 2.500Xg for 15 minutes to pellet the cell debris.

8- Transfer supernatant (500ml) to a new cold tube for add Laemmli sample buffer (500ml) and shake up and down gently five times.

9- The total cell protein should be rapidly store frozen to avoid protease degradation of proteins or collected and lysed.

3.4.3. Gel and Solutions

There are some gels and solution should prepered before start working like

1- Separating gel.

2- Stacking gel.

3- Luminol solution.

4- Hydrogen Peroxide Solution.

5- 10X SDS Running buffer.

6- 10X Western Blot Transfer Buffer.

7- 10X TBS pH 7.4.

8- Sample Buffer (Laemmli buffer).

9- RIPA buffer (pH 7.4± 0.1).

3.4.4. Procedure

3.4.4.1. Preparation of Poly Acrylamide Gel

1- Put the comb between the glass plates and make a line on the class 1cm below the teeth of the comb. This will be the level between two gels then remove the comb.

2- Make of 10 ml separating gel. Immediately mix and use pipette to pour the solution between the gel plates.

3- Put on 1-2 cm isopropanol onto the surface of the gel solution to prevent exposure to the air. Leave the gel to set and wait polymerization, discart the isopropanol.

4- When the gel has set pour off the alcohol and rinse the surface of the gel with water in one side, Use a piece of filter paper to remove excess water.

5- Prepare a 5% stacking gel. Mix all of them immediately.

6- Insert the comb (Half) at an angle to a void trapping air bubbles under the teeth of the comb. Leave the gel to set. Sweep any extra gel immediately (wait 10-20 minute).

3.4.4.2. Protein Separation (SDS-PAGE)

1- Boil the samples for 4 minutes at 96°C.

2- Transfer 10ul of each sample to SDS-electrophoresis.

3- Prepare Marker sequence.

4- Air bubbles get trapped under the bottom of the gel.

5- Flash the wells out with running buffer.

6- Load the samples on the polyacrylamide gel.

7- Run the gel at the 25A for 25- 40 minutes until the samples reach the line between stacking gel and polyacrylamide gel.

8- Switch the 25A to 40A and wait 50 minutes or the sample down in the base.

3.4.4.3. Western-Blot Method

The western (note that in this context "western" should be splet with a lower-case blot is commonly used to identify, quantify and determine the size of specific proteins. The procedure of SDS-PAGE is based on the characteristics of SDS, which is a strongly anionic detergent.

Because proteins do not all have the same electrical change, the mixture is treated with SDS and thus the proteins become denatured and negatively changed. As a result, this allows separation of proteins by moleculad weight. Immediately after the proteins have run to the bottom of the gel, electro-blot them on to a nitrocellulose membrane into Transfer Buffer. 40Mm Tris, 40mM glycine, 0.0375% (v/v) SDS, 20% methanol. The separated proteins were transferred from within the gel onto a membrane with 170Ma currency electric field for 110 minutes. The effectiveness of protein transferring was determinate with staining Ponso-S for 1 minute and then washed with TBS-T.

Setting up Western blot transfer using BioRad Mini-Trans apparatus. Wearing gloves, cut the membrane and the filter paper to the dimensions of the gel. Equilibrate the gel in transfer buffer (room temperature) for 30 minutes. Using methanol in the transfer buffer is important as the methanol will cause the gel to shrink and cause distoration in the transfer process. Also soak the membrane, filter paper and fibre pads in transfer buffer. The nitrocellulose membrane draw grid by pencil to indicate the region. Prepared the gel-membrane-papers sandwich was place into the cassette.

Then cassette inserted into equipment for transferring. The orientation of the cassette so its grey side in facing the similarly rey colored well of the electrode module. Repeat for the second cassette. While transferring performed the buffer should be cool saved with frizen accumulator. The block non-specific sites by soaking in 5% BSA in TBS-T (0.5-1 hr, RT). After transferring turn off the power and remove membrane from the sandwich.

3.4.4.4. Incubation with Antibodies

A. Primary antibody -CD56/NCAM-1

Washing by TBS-T 5 minutes, 5 minutes and 5 minutes at room temperature with moving. Block the membrane in TBS-T + 5% by incubating with moving at 37°C. Weigh 0.5g of bovine albumin and add to TBS-T 15ml with moving gently for ten minutes by hand gently to avoiding any papoles and after that divide the 15 ml into two centrifuge tube 7.5ml for each one.

Finally add 2.77 µl of anti CD56 antibody to each one and move gently. Put the membrane in the solution and close the tube lid by parafilm. Incubate the membrane with the diluted primary antibody at 4°C for an overnight.

Incubate with primary antibody was performed with 1:5000 of antisera disolved in BSA/TBS-T. After 12-14 hours incubation at 4°C were wash three times with TBS-T (3 x 5 minutes).

B. Secondary antibody Rabbit HRP N2013

The membrane was incubated with the anti-Rb HRP (Abcam, ab6721) antibody solution for 60 minute at the room temperature under constantly rotation. The membrane was incubated with the dilution secondary antibody at 4°C for an overnight in BSA /TBS-T. 0. After 12 – 14 hours incubation at 4°C the membranes were washed three times with TBS-T (5 x 5 minutes).

3.4.4.5. Detection and Quantification

Detection methods of chemiluminescent were used most ofter and therefore enhanced chemiluminesence (ECL) is a sensitive method and can be used for relative quantitation of the protein of interest.

The primary antibody binds to the protein of interest and the secondary antibody usually linked to horseradish peroxidase that used to cleave a chemilumenescent agent.

The create of luminescent signal need incubate the membranes for 3-5 minutes with shaking in a dark place in a solution that contain luminal solution and hydrogen peroxide solution. The developing film (Konica Minolta MG, Japan) staining should be performed with X-Ray machine. Detect the signal on X-Ray film for scan the developed film and quantify the signal by using image analysis software.

Once an image is achieved from the blot, it can be analyzed by densitometry to measure the relative amount of a specific protein on the blot by comparing it with a control or specific time point.

This quantification is necessary, if a researcher wants to compere samles. It is omportant to attempt to achieve an exposure of the image where the bands are sharp (not fruzzy or indistinct edges). If sample are being compared, the ideal situation is to run all samples on one blot, because there can be variation between blots.

3.5. CBC test (complete blood count)

The instrument system is Medonic M-Series Hematology-Sweden.

3.5.1. Principle of Test

The method of measuring the size and counting particles uses measurable changes in electrical resistance produced by nonconductive particles suspended in an electrolyte. A suspension of blood cells passes through a small orifice simultaneously with an electric current. A small opening between electrodes is the sensing zone through which suspended particles pass. In the sensing zone, each particle displaces its volume of electrolyte.

3.5.2. Specimen Collection

Three ml of venous blood was collected and added directly into K3 EDTA tube (a 1-2% dilution effect occurs in this liquid EDTA tube) with rotation by the coulter.

3.5.3. Specimen Preparation

1- The blood sampls were immediately used to run the CBC after being collected. Collected blood samples in an EDTE K3 tubes were inverted it 8-10 times to mix blood and the anticoagulant. The samples were mixed for 10-15 minutes before analysis.

2- Preparation of reagents, calibration (standards), controls, materials, equipment and instrument.

3.5.4. Evaluation

CBC evaluates three types of blood cells.

1- White blood cells (WBC) count. WBC is a count of the total number of white blood cells in a person's blood. It identifies and counts the number of the different types of white blood cells available. The types include neutrophils, lymphocytes, monocytes, eosinophils and basophils.

2- Evaluation of red blood cells. Red blood cell (RBC) count is a count of the number of red blood cells in a person's blood. Hemoglobin reflects the number of red blood cells in the blood, Hematocrit measures the percentage of a person's total blood volume consists of red blood cells. Mean corpuscular volume (MCV) is the average size of a single red blood cell. Mean corpuscular hemoglobin (MCH) is the average amount of hemoglobin inside a single red blood cell. Mean corpuscular hemoglobin concentration (MCHC) is a calculation of the average concentration of hemoglobin inside a single red blood cell. Red cell distribution width (RDW) is the variation in the size of RBCs and also include reticulocyte count.

3- Evaluation of platelets. The platelet count is the number of platelets in a person's blood. Mean platelet volume (MPV) is a calculation of the average plateletes size. Platelet distribution width (PDW) reflects uniform platelets are in size.

3.6. Statistical Analysis

Statistical analysis were conducted to describe different variables and parameters in this research and to describe relationships with each other as well.

1- The research was used to find out the significant of differences between the age and gender of patients and control group.

2- The research was used to Elucidate of molecular mechanisms that induce the disturbance of progress of cancer and immune response.

3- PCR technique, Western blotting and CBC test were used to find out the effect of different patients criteria on the reading of EBER-2 gene, CD56 marker in blood cells.

4- Quantitative results were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests. P values <0.05 were considered to indicate statistical significance. SPSS version 12.0 was used for the statistical analyses.

4. RESULTS

Over a period of 3 months 40 blood samples were collected from cancer patients and also from healthy control individuals, including (25) females (62.5 %) and (15) male (37.5 %), as shown in figure 4.1, attending Hiwa hospital in Sulaimaniyah province.

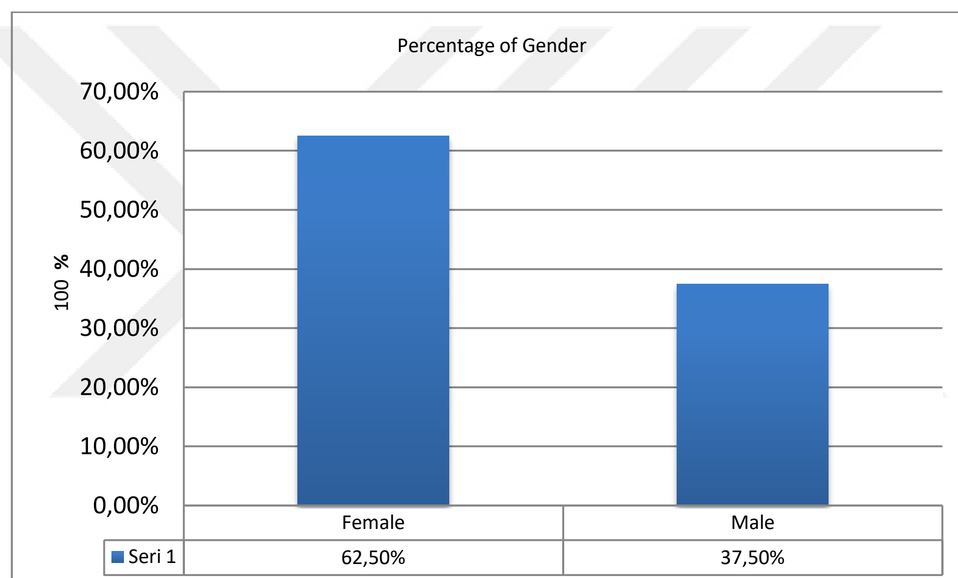

Figure 4.1. The percentage of Female and Male examined

The blood samples were collected from (11) females between (25 to 35) years old were highest number compered with another females and mans ages, (6) females between (35 to 45) years old, (8) females between (45 to 55) yers old, (4) males between (25 to 35) years old, (3) males between (35 to 45) years old were lowest number compered with another females and mans ages and (8) males between (45-55) as shown in figure 4.2.

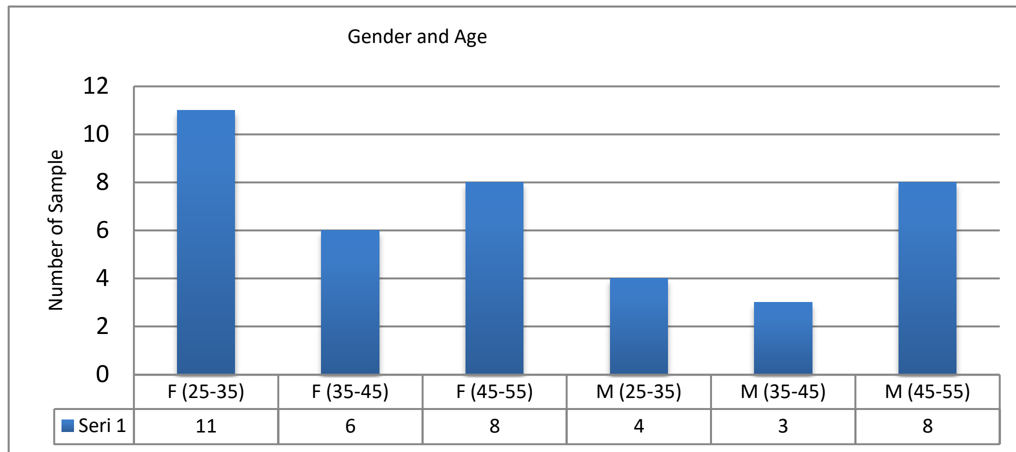

Figure 4.2. Total number of Gender and Age of Cancer patients and normal control F: Female, M:Male

The forty blood samples are gathered form four groups (Control Group, Nasopharyngeal Carcinoma, Gastric Carcinoma and Breast Cancer). The number of Control healthy group patients was 10 (Male: 5, Female: 5), the number of Nasopharyngeal Carcinoma patients was 6 (Male: 5, Female: 1), the number of Gastric Carcinoma patients was 9 (Male: 5, Female: 4) and the number of Breast cancer patients was 15 (Male: 0, Female: 15), as shown in Figure 4.3.

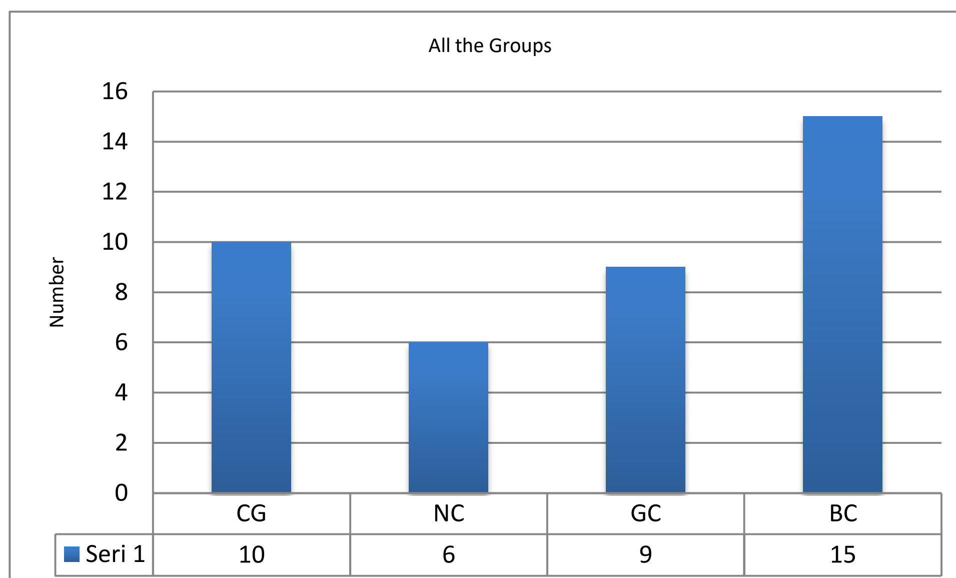

Figure 4.3. The Numbers of Persons were shared in all the Groups CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

4.1. Standard PCR Results

The blood samples were examined by standard PCR assay using 2 primers, one of them specific for a known mutation (EBER-2), the percentage rate of EBER-2 mutation gene in the samples were (66.6%) in the patients with Nasopharyngeal Carcinoma, (50%) patients with Gastric Carcinoma and (33.3%) patients with Breast Cancer and (30%) in the healthy control group as shown in the figure 4.4.

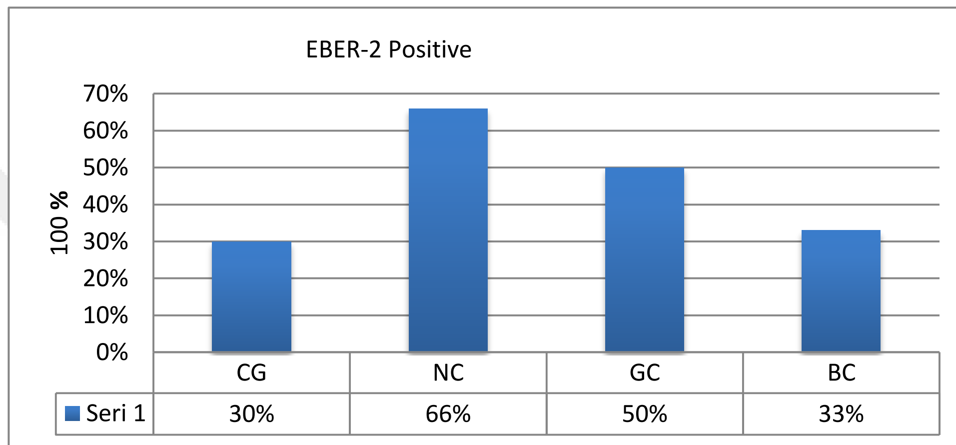

Figure 4.4. The percentage of positive EBER-2 Gene detected by standard PCR in all the groups
CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

Seventeen samples were positive with EBER-2 gene mutation detected by standard PCR technique, (14) patients and (3) control out of forty as shown in figure 4.5.

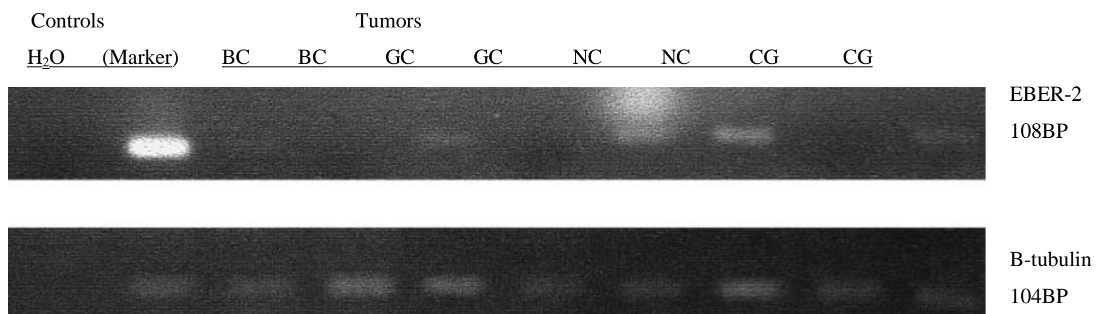

Figure 4.5. Detect Epstein-Barr Virus genome by PCR amplification. The 108-bp EBV-encoded small RNAs (EBER-2) fragment. A 104-bp b-tubulin sequence was amplified to verify the presence of genomic DNA in each sample extraction. H_2O water control without DNA and (+)

= positive control. CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer.

4.2. RT-PCR Results

Through testing 20 blood samples by Real-time PCR were collected from cancer patients and also from healthy persons as control and each group has 5 persons. Table (4.1) include (15) female (75 %) and (5) male (25 %) in Hiwa hospital. Most of this samples that have all four groups (Nasopharyngeal Carcinoma, Gastric Carcinoma, Breast cancer, Control), the percentage rate of available of Epstein –Barr virus gene depending on the QIAGENE company kit, samples were (100%) in the indivedual have Nasopharyngeal Carcinoma, (80%) in the persons have Gastric Carcinoma, (60%) in the persons have Breast cancer and (80%) in the control healthy group as shown in the figure 4.6.

Table 4.1. Quantitative analysis of cycling A.Green

Number	Type	Result (Positive, Negative)	Result (Copy/ml)
No1	Nasopharyngeal C.	+	8099000
No2	Nasopharyngeal C.	+	2080000
No3	Breast C.	-	0
No4	Breast C.	+	8097000
No5	Breast C.	+	205000
No6	Gastric C.	+	22426000
No7	Nasopharyngeal C.	+	158148000
No8	Gastric C.	+	632831000
No9	Gastric C.	+	7149000
No10	Gastric C.	+	688000
No11	Gastric C.	-	0
No12	Breast cancer	+	856000
No13	Nasopharyngeal C.	+	26781000
No14	Nasopharyngeal C.	+	551034000
No15	Control	-	0
No16	Control	+	2345000
No17	Control	+	6789000
No18	Control	+	43210000
No19	Breast C.	-	0
No20	Control	+	838000

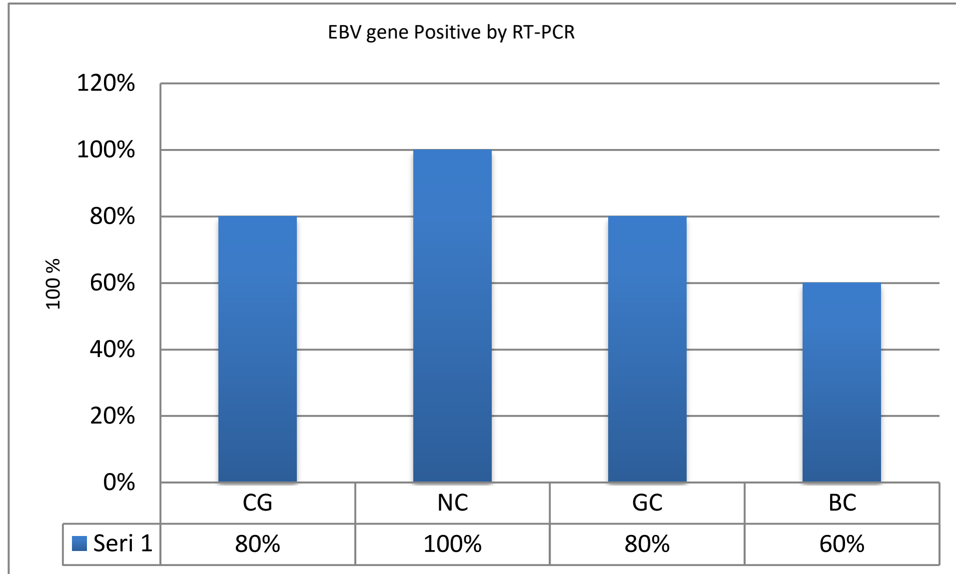

Figure 4.6. The Percentage occurrence of EBV Gene detected by RT- PCR in all the groups CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

4.3. The Role Expression of Marker CD56 in Leukocytes Samples from Blood of Control and atients Groups

The role of CD56 had revealed that higher percentage of patients have positive cases and detected with EBV or with negative cases compering with healthy control that has low perecent as shown in table (4.2), that most our positive and negative EBV genes in patients groups (Breast cancer, Gastric carcinoma, Nasopharyngial carcinoma) were in averages of (184,144,141) in respectively and the average of healthy control was (102) as shown in Figure (4.7).

The resuts of CD56 study in the samples from the blood of patients shown statistically differences between control and all cancers groups. The observed differences were more significant into Breast cancer group and it average (82%) For another groups especially gastric carcinoma group and Nasopharyngeal carcinoma group average were (45%) and (39%) accordingly.

Table 4.2. The result of Anti-CD56 by Immunoblot method to all the samples

Control Group	Breast Cancer	Gastric C.	Nasopharyngeal C.
92	207	167	140
95	169	189	124
93	167	118	142
91	163	94	121
155	139	167	162
100	163	151	157
97	179	124	142
100	221	140	140
100	202	151	141
102	208	162	141

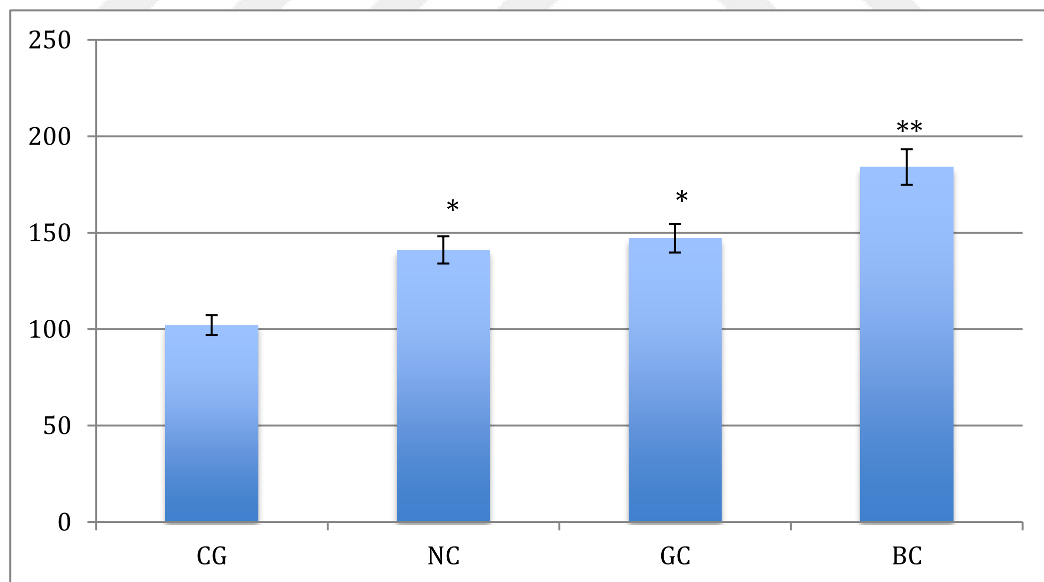

Figure 4.7. The rates of Anti-CD56 by Immunoblot method and related with all groups CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

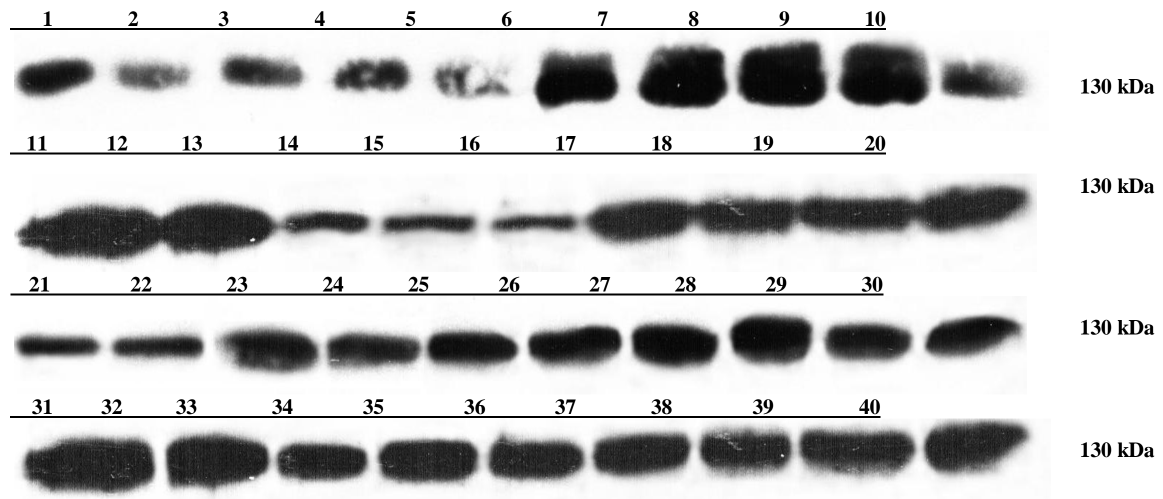

Figure 4.8. Detection of Anti-CD56 by Immunoblot method in all the groups (130 kDa)
11,21= Marker 2, 3, 4, 5, 14, 15, 16, 22, 23=Control Group 6, 7, 8 , 9, 10, 12, 13, 19, 24, 26, 27, 28, 31, 32, 39= Brest Cancer 18, 20, 25, 29, 30, 33, 34, 35, 36, 37= Gastric Carcinoma 1, 17, 38, 40=Nasopharyngeal Carcinoma

4.4. Distribution of Complete Blood Count with Cancer Cases and Compare with Control Group.

The CBC test is a panal for evalution of three types of cells in the blood (white blood cells (WBC), Red blood cells (RBC) and platelets (PLT) that is associated with the different types of cancer through the decreasing and increasing the counts of blood cells. CBC test is widly used for individuals who have cancer for all the ages and genders compering with the healthy control groups.

In the figure (4.9) shown the results of CBC tests demonstrated that in the patients have Breast cancer, the average of HGB was (12.2 g/dl), WBC (6,700 10^9/1), PLT (292 10^9/1), MPV (7.6 fl), Lymphocyte cells (1.8 %) and the average of Granulocyte cells was (4.17 10^9/1).

Also, the figure (4.9) bored the results of CBC test cleared that in the patients with Gastric Carcinoma groups, the average of HGB (12.2 g/dl), WBC (5,400 10^9/1), PLT (186 10^9/1), MPV (8.3 fl), Lymphocyte cells (1.7 %) and the average of Granulocyte cells was (3.2 10^9/1).

The results of CBC tests are confirmed that in Nasopharyngeal Carcinoma group of patients, the averages of HGB was (13.3 g/dl), WBC (4,800 10^9/1), PLT (271 10^9/1), MPV (7.8 fl), Lymphocyte cells (1.2 %) and the average of Granulocyte cells was (3.4 10^9/1).

Finally, there are determined the results of the healthy control group through indication the evarege of HGB (15.1 g/dl), WBC (7.100 10^9/1), PLT (219 10^9/1), MPV (8.9 fl), Lymphocyte cells (2.1 %) and the average of Granulocyte cells was (4.3 10^9/1) as shown in Figure 4.9.

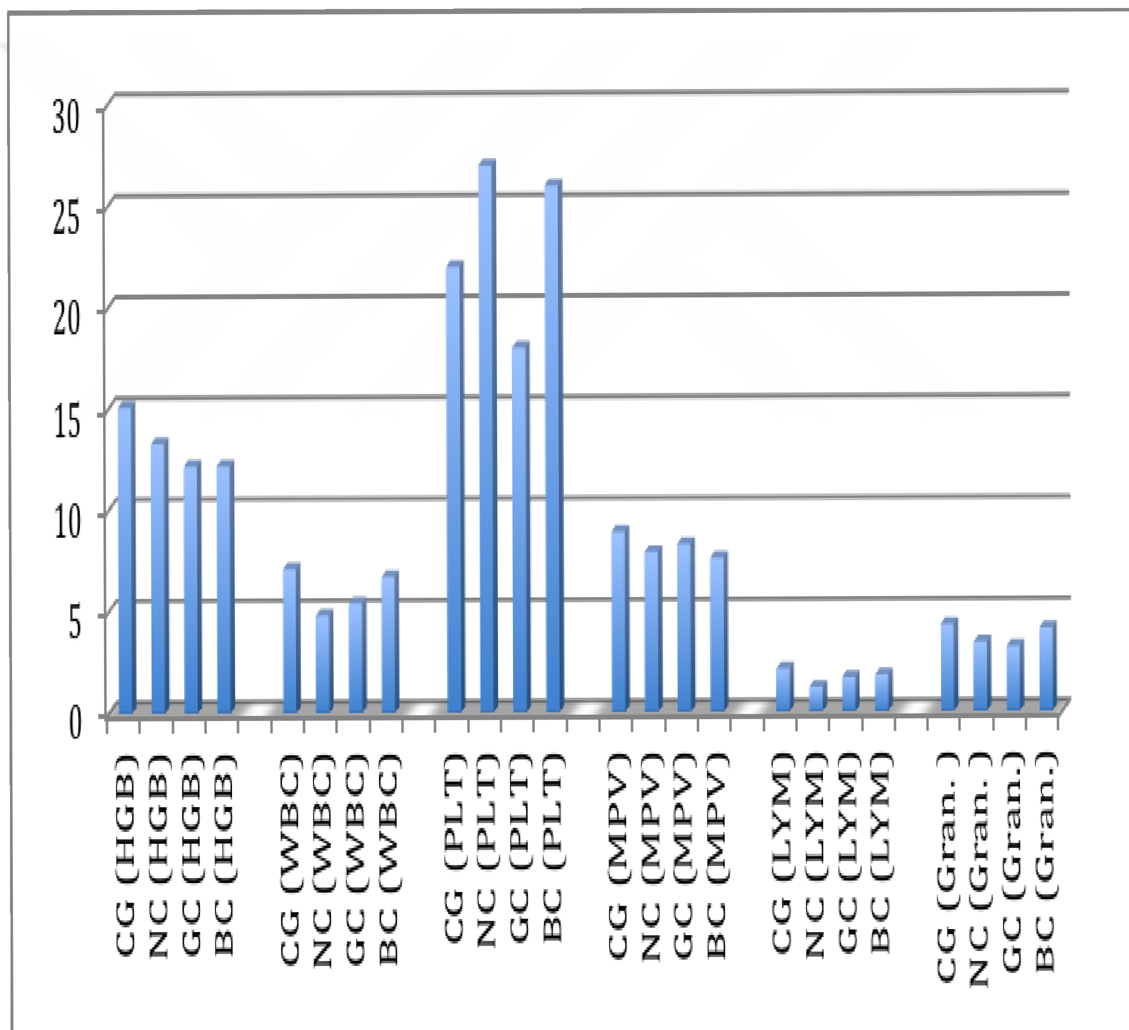

Figure 4.9. The results of CBC Test in test in the blood of patients with all groups Unit (HGB: g/dl, WBC: 10^9/1, PLT: 10 ^9/1, MPV: fl, Lym: %, Gran: 10 ^9/1)

The resuts of HGB study in the samples from the blood of patients show different statistics between control and all cancers groups. The observed differences were more significant into Breast Cancer group and Gasteic Carcinoma there are average (2.9). For another groups especially Nasopharyngeal Carcinoma average (1.8) accordingly to compare with control group average as shown in the Figure 4.10.

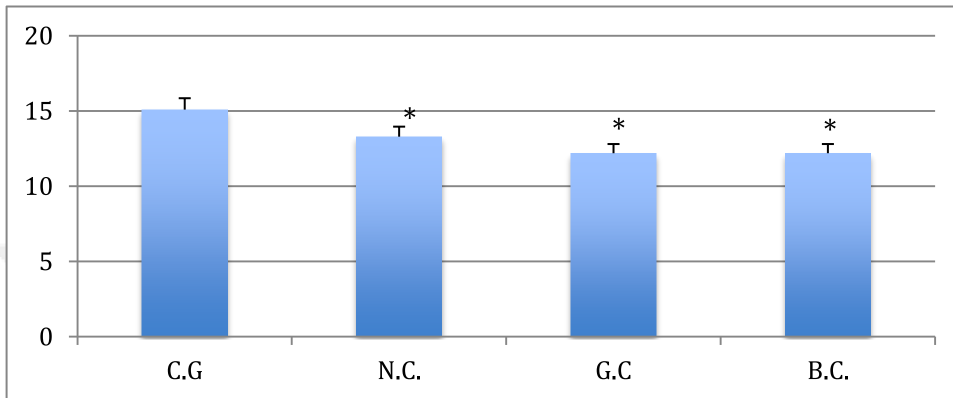

Figure 4.10. HGB contents in control and related with Cancer groups (Unite: g/dl) CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

The resuts of WBCs shown statistically differences between control and all cancers groups. The observed differences were more significant into Nasopharyngeal Carcinoma group and it average (2,300) for another groups especially Gastric Carcinoma (1,700) and Breast Cancer average (400) accordingly, when compared with control group and shwon in figure 4.9.

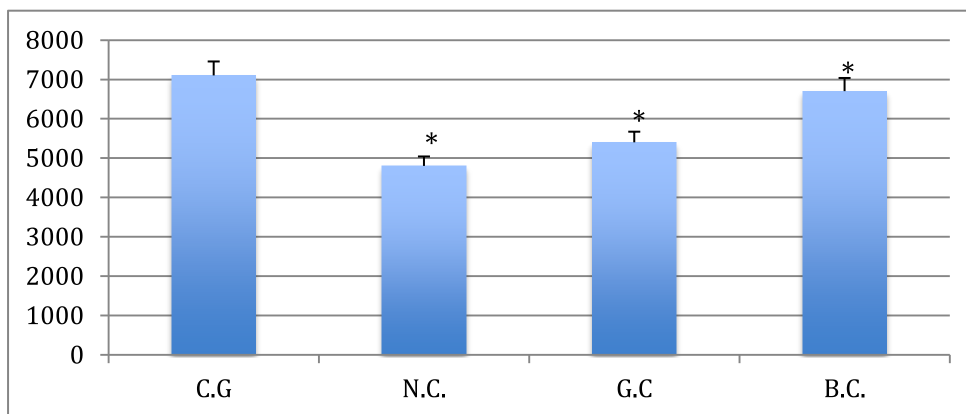

Figure 4.11. WBC contents in control and related with Cancer groups (Unit: 10^9/1) CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

The resuts of PLTs shown statistically differences between control and all cancers groups. The observed differences was significant into Gastric Carcinoma group and it average (27) less the control average and another groups were highest especially Nasopharyngeal Carcinoma average (52) and Breast Cancer (43) accordingly, when compered with control group as shown in Figure (4.12).

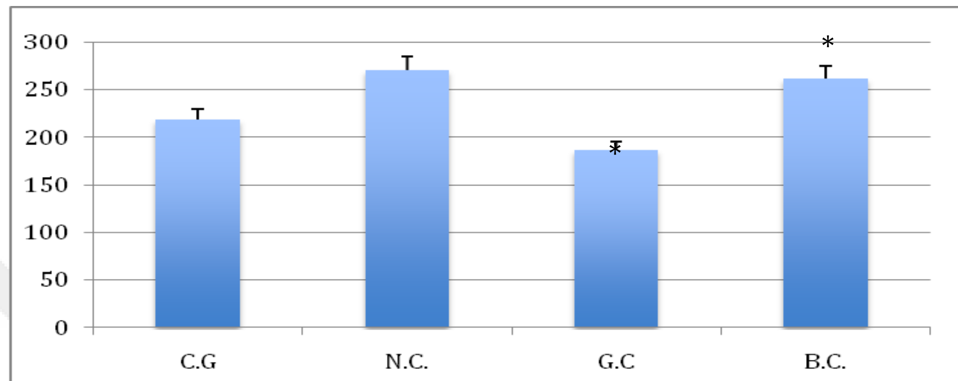

Figure 4.12. PLT contents in control and related with Cancer groups (Unit: 10^9/1) CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

The resuts of MPV tests showns statistically differences between control and all cancers groups. The observed differences were more significant into Breast Cancer group and it average (1.3) For another groups especially Nasopharyngeal Carcinoma (1.1) and Stomach Cancer average (0.6) accordinaly comparing with control group and it shown as Figure (4.13)

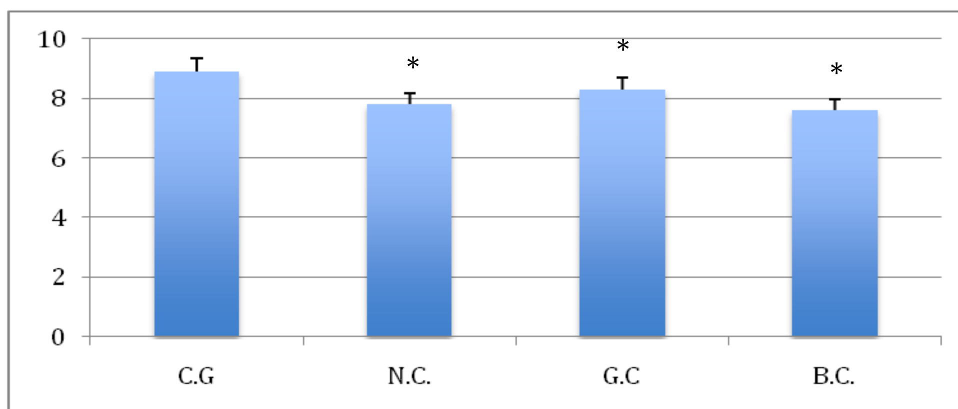

Figure 4.13. MPV contents in control and related with Cancer groups (Unit: fl) CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

The resuts of LYM showns statistically differences between control and all cancers groups. The observed differences were more significant into Nasopharngeal carcinoma group and it average (0.9) For another groups especially Stomach cancer average (0.4) and breast cancer (0.3) accordinaly, as shwon in figure 4.14.

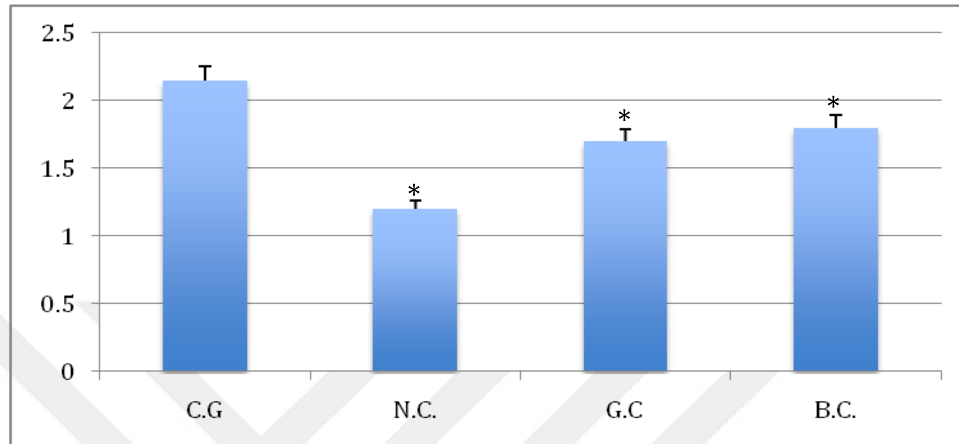

Figure 4.14. LYM contents in control and related with Cancer groups (Unit: %) CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

The resuts of Granulocyte tests showns statistically differences between control and all cancers groups. The observed differences were more significant into Gastric carcinoma group and it average (1.1) For another groups especially Nasopharyngeal carcinoma average (0.9) and breast cancer (0.2) comparing with the control group (4.3) and according to Figure 4.15.

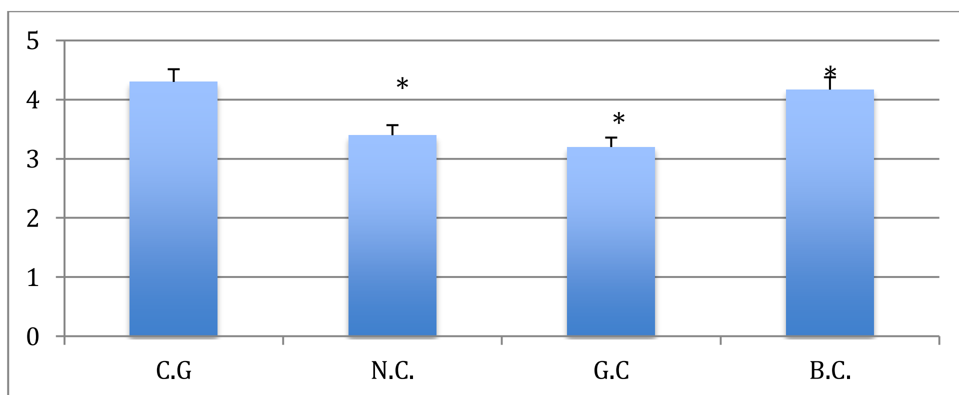

Figure 4.15. Granulocyte contents in control and related with Cancer groups (Unit: 10^9/1) CG: Control Group, NC: Nasopharyngeal carcinoma, GC: Gastric Carcinoma, BC: Breast Cancer

5. DISCUSSION

In this research both the women and men had deffirent types of cancers and it was clearly Increased percentage for a women group more than it observed for the men group (figure 4.1). There might by because of the natural living environment and the case of each patients that supported with Joshi et al 2009. Once cause that is women have joined a male workplace and changed habits when exposed them to both industrial and environmental carcinogens such as cigarette smoking from which they previously had been excluded. It is possible that genetic hormonal, anatomical or other factors may explain this puzzling trend. Concerning age distribution the present study has observed that most of the patients with cancer disease and control samples were in the age range of (25- 55) years and divided to females and males as shown in Figure (4.2) and demonstrated that most of females patients in have Breast cancer between 25 to 35 years and the rest female groups between 35 to 45 have 6 persons and between 45 to 55 years. In male patients these results are in accordance with the studies of other researchers (Thorley et al. 2004). The results in figure (4.2) of the current study cleared that the age of cancer patients samples were between 25 to 55 years and was in agreements with other studies from (Odumade et al. 2011), who reported that the cancer with EBV usully develop between the age of 25 to 60 years. The increasing incidence with age may be explained by the accumulation of somatic mutations associated with the emergence of malignant neoplasms. In addition, the observed impairment in the immune system in such ages, due to decline in the immune surveillance which might lead to accumulation of cellular DNA mutation that could be regarded as an additional significant factor in the development of such malignancies (Burns and Leventhal 2000). Also decline in the number of NK may play an important role in early natural-surveillance against cancer and infectious disease, a progressive age related shift in the circulating lymphocyte population from conventional T cells to NK cells (Ravaglia et al. 2000).

EBV was the first human virus directly connected with carcinogenesis. Most humans live with the virus without serious risks. A small number of these infected individuals may develope tumors as EBV has been implicated in the pathogenesis of lymphoma, carcinoma in immunocompromised individuals. The presence of this virus has also been associated with epithelial malignancies arising in the gastric region and the breast. EBV has more viral proteins and work as anti-apoptotic factors, to usurp control of the cellular pathways and regulate diverse homeostatic cellular functions. In this study, association between EBV and all patients was examined by standard PCR that demonstrated EBV was observed in about 55% of patients in figure (4.4) and was also demonstrated by Quentititive PCR method in about 84% (Table 4.1) and figure (4.6). That result was in agreement with the finding of (Matthew and Razelle 1994) which divided the cancer patients to subgroup.

Undifferentiated nasopharyngeal cancer affects mainly individuals in their patients and is more common in men and the age between 40-60 years old (Vasel et al. 1997) as we shown in this research the Nasopharyngeal Carcinoma group were mankinds (83%) and old age. In this research, investigated the association between EBV and Nasopharyngeal Carcinoma detected by Standard PCR test results which demonstrated EBER-2 was observed in 66% of patients shown in Figure (4.4) and demonstrated in table (4.1) and figure (4.6) that EBV gene in Quentititive PCR test was 100%. This result was in agreement with the finding of (Eduardo et al. 2010) which detected the EBV genome in 84% of male and 15% of female in peripheral blood samples. NPC has been demonstrated to be an Epstein-Barr Virus-associated cancer. The circulating tumour DNA concentration in the blood of patients with NPC can be quantified using real-time quantitative polymerase chain reaction (PCR) (Wang et al. 2011).

Epstein-Barr Virus-associated gastric carcinoma is a distinct subtype and defined by monoclonal proliferation of carcinoma cells with latent EBV infection demonstrated by EBV-encoded small RNA (EBER) by using PCR methods (Masashi et al. 2014). In this study, the association between EBV and Gastric Carcinoma was investigated using standard PCR that demonstrated that EBV was observed in nearly 50% of patients shown in figure (4.4). This result was in agrement with the finding of (Matthew and Kurzrock 2004) which detected the EBV genome in about 50% of male and 15% of female and must of the ages patients above 40 years old like the result of Busson et al. 2009).

Breast cancer is the most common cancer among women compered with the anther kinds of cancer, it is increased clearly of the number of patient every years and may leading to death (Joshi et al. 2009). Considering of the role for positive EBV in the development of Breast Carcinoma may have impacts on prevention and early detection of the Breast Cancer in infected and high risk populations in future (Mohammadizadeh et al. 2014).

The identification of EBV genome in breast carcinoma and the role as a carcinogen has been always discussed despite of many studies tha well documented the presence of EBV genetic material in up to 51% of breast tumors (Murray and Young 2002). The present study demonstrated the presence of EBV in 33% of breast carcinoma samples by PCR amplification as shown in figure (4.6). This result was in agrument with the finding of (Perkins et al. 2006) which detected the EBV genome including the relative proportion of positive cases 29%.

EBV genes may stay in the cells of the body without causing the appearance of any signs of viral infection and remain in the individuals as latent stage. In this research, the association between EBV and control individuals was investigated using standard PCR and Real-time PCR to quantitate EBV genomes in the peripheral blood mononuclear cells. The results showed that about 30% positive as shown in Figure (4.6). This result was in agreement with study of healthy individuals who reported a viremia detection rate of 27% by means of conventional PCR (Gan et al. 1994).

Increase of CD56 marker in healthy individuals are rare but it can be clear detected in different tumor cases and it can be classified into natural killer (NK)-like T-cell lymphoma or NK cell lymphoma and different plasma cells (Pernick 2015). The test of CD56 was done by immunoblot method of three patients groups and control in this study and shown that most cancer patients are CD56 positive with NK-like T-cell lymphoma and confirmed the presence of increasing numbers of CD56 in patients with breast cancer, then Gastric carcinoma and Nasopharyngeal carcinoma decreases as shown in the figure (4.7) Breast carcinomas with advanced stage disease and showed aggressive behavior. With useing of specific markers is thus encouraged in this subtype to enhance detection of differentiation and hence characterize the biological and therapeutic (David et al. 2014). These results suggest that NK cells and NK-like T-cell lymphoma pursue an aggressive clinical course. EBER-2 and PCR gene rearrangement are useful in

distinguishing NK cell lymphoma from NK-like T-cell lymphoma, particularly when blood samples were tested directly.

In this research, we see clearly the reduction in the rate of normal blood cells in most patients cancers like as shown in figure (4.9) because of the therapies were used to damage or kill cancer cells and most of the side effects of cancer treatment began by the harm to normal cells only last for a short time, so the immune cells of cancer patients have been shown to be impaired in their ability to produce tumor necrosis factor as saported from (Zielinski et al. 2003). Higher normal numbers of lymphocytes or monocytes can indicate the possibility of certain types of cancers and their cancer treatments may cause neutropenia and may cause a decrease in red blood cells, also some results may cause a decrease in platelets and that results saported by (Campbell et al. 2005). The CBC test results have many factors containing non cancerous conditions; can lead to results that fall out of the normal range or to lead diseases.

CONCLUSION

The cancer types with EBV invasion was the commonest in patients with progress of cancer and immune defect. There were found significant correlation among these factors. Among the forty cases (84%) were found as EBV positive. The rest of patients (16%) were EBV negative determined with RT- PCRmethod. Expression rate of EBER-2 gene of EBV DNA by conventional PCR method was 66% in Nasophergeal carcinoma group while low percentage of it in Gastric Carcinoma cases (50%) and (33%) positive founds in breast cancer. The overall expression of EBER- 2 in healthy control cases in this study was 30% it means evident there was significant correlation between them. Strong correlation rate was observed between the cancer cases and immune defect with CD56 expression as a cancer marker in the CD56 content was signifisantly increased in all cancer groups compaired with normal healthy control. The results of CBC tests were associated with cancer cases and overt the decrease of the level blood cells.

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