UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

# DETECTION AND CHARACTERIZATION OF EPSTEIN BARR VIRUS IN NASOPHARYNGEAL CANCERS IN GHANA

BY

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# DECLARATION

I, Richmond Ayee hereby declare that this thesis is the outcome of my own research project under the supervision of Dr. Osbourne Quaye of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana and Dr. Edward Wright of the Department of Biochemistry, University of Sussex, Brighton, U.K. To the best of my knowledge, this thesis has not been presented for the award of any degree or published elsewhere. Any mention of other authors' works has been duly acknowledged and properly referenced.

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(Supervisor)

# DEDICATION

I dedicate this research work to my mother, siblings and my entire family for their prayers and support for me day in and day out.

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# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **NPC** | **: Nasopharyngeal cancer** |
| **EBV** | **: Epstein Barr Virus** |
| **BL** | **: Burkitt's lymphoma** |
| **HL** | **: Hodgkin lymphoma** |
| **BC** | **: Breast cancer** |
| **GC** | **: Gastric cancer** |
| **PTLD** | **: Post-transplant lymphoproliferative disorder** |
| **EBVaGC** | **: Epstein Barr associated gastric cancer** |
| **EBNA** | **: Epstein Barr nuclear antigen** |
| **LMP** | **: Latent membrane protein** |
| **ENT** | **: Ear nose and throat** |
| **PCR** | **: Polymerase chain reaction** |
| **PBMC** | **: Peripheral blood mononuclear cells** |
| **ORF** | **: Open reading frame** |
| **$C_T$** | **: Cycling threshold** |
| **VCA** | **: Viral coat antigen** |
| **EBER** | **: Epstein Barr encoded RNA** |
| **IHC** | **: Immuno histochemistry** |
| **HHV-4** | **: Human gammaherpes virus 4** |
| **KBTH** | **: Korle-Bu Teaching Hospital** |
| **WHO** | **: World Health Organization** |
| **EA** | **: Early Antigen** |

# ABSTRACT

**Purpose**: Nasopharyngeal cancer (NPC) is a malignant tumor which is commonly associated with Epstein Barr virus (EBV). Two genotypes of EBV, genotype 1 and genotype 2, which are geographically restricted, are implicated in the pathogenesis of NPC. This study aimed at detecting and characterizing EBV genotypes involved in the pathogenesis of NPC in Ghana.

**Experimental design**: Whole blood and biopsy samples were collected from 55 patients diagnosed of NPC by CT scan and endoscopy at the Ear, Nose and Throat Unit (ENT) of the Korle-Bu Teaching Hospital (KBTH). As control subjects, whole blood was collected from 53 individuals confirmed NPC negative or without known oncological diseases by CT scan and endoscopy. Association of NPC with risk factors such as gender, alcohol intake, smoking and consumption of salted fish, was evaluated. EBV was detected by amplification of Epstein Barr nuclear antigen 1 (EBNA-1) and genotyping by amplification of Epstein Barr nuclear antigen 2 (EBNA-2) using primers specific to genotypes 1 or 2. Viral load in blood and biopsy specimen was quantified using real-time polymerase chain reaction (PCR).

**Results**: Risk factors such as gender, consumption of alcohol and smoking were not significantly ($p > 0.05$) associated with NPC development. There was a significant association ($p< 0.05$) of consumption of salted fish with NPC development. The frequencies of EBV positivity were 67%, 67% and 92% in NPC blood, NPC biopsies, and control blood samples, respectively. The predominant EBV genotype in blood specimen from cases was EBV genotype 2 (52%) and that of the control group was type 1 (62%). Statistically significant difference ($\chi 2= 72.26$, $p = 0.001$) was observed for the frequency of EBV genotypes in the NPC blood and controls. In biopsy specimen, the predominant EBV genotype was genotype 1 (58%). Median viral load ($9\times10^7$ copies/ mL) in whole blood of NPC case subjects was significantly higher than median viral load ($6\times10^4$ copies/ mL) in the whole blood of control subjects ($p=0.001$). Significantly higher ($p < 0.001$) median EBV DNA load ($9\times10^7$ copies/ mL) was observed in

NPC whole blood samples compared to that of NPC tumor biopsies ($1.58\times10^5$ copies/mL). The median viral load of case samples infected with EBV genotype 1 was significantly higher ($p = 0.001$) than control samples infected with the same EBV genotype (genotype 1 median viral load in cases, $2\times10^7$ copies/ mL verses $29\times10^3$ copies/ mL genotype 1 median viral load in controls). Significantly higher ($p = 0.001$) median viral load was observed in cases infected with EBV genotype 2 when compared to control samples infected with the same EBV genotype ($1\times10^8$ copies/ mL compared to $18\times10^3$ copies/ mL in cases and controls, respectively).

**Conclusions**: In summary, gender, consumption of alcohol and smoking were not associated with NPC development in the current study whereas association was established between NPC and salted fish consumption. The frequency of EBV was higher in control subjects than NPC patients, confirming reports suggesting large number of the world’s population being asymptomatic carriers. Gender was not a risk factor of EBV infection in this study. This study identified EBV genotype 2 infection as the virulent genotype in Ghana and a possible risk factor in the development of NPC in Ghanaian patients. EBV genotype 1 was predominant in the control group and consistent with the genotype being relatively less virulent. Detection and quantification of EBV load can be used as a non-invasive biomarker for diagnosis of NPC.

# CHAPTER ONE

## 1.0 INTRODUCTION

## 1.1 BACKGROUND

Nasopharyngeal cancer (NPC) is a malignant tumor which occurs at the back of the nasal cavity (Banko *et al.*, 2016). Nasopharyngeal cancer is uncommon in most populations around the globe but has a high incidence in South-East Asian countries. The disease is rare in Africa, however, there have been reported cases of NPC in Ghana and other Africa countries such as Algeria, Tunisia, Sudan (Adam *et al.*, 2014; Breda *et al.*, 2010). In Ghana, NPC is the second leading cancer disease of the Head and Neck with a prevalence of about 1.2 % at the Ear, Nose and Throat (ENT) unit of the Korle Bu Teaching Hospital in Accra (Kitcher *et al.*, 2004). The variation in ethnic and geographical distribution of NPC indicates a multifactorial etiology, involving genetic susceptibility, Epstein Barr virus (EBV) infection, ethnics, environmental factors and food consumption (Brennan, 2006; Li *et al.*, 2008).

The most common etiological factor associated to NPC development is EBV infection. Human gammaherpes virus 4 (HHV-4) or EBV is a ubiquitous oncogenic virus belonging to the family *Herpesviridae (Chang et al., 2009; Schafer et al., 2015)*. The *Herpesviridae* family is further classified into the subfamilies alpha, beta, and gammaherpes virus. Epstein Barr virus is a classical member of the subfamily *Gammaherpesvirinae* and among nine viruses that have been identified to solely infect humans (Longnecker *et al.*, 2013; Sathiyamoorthy *et al.*, 2016). The virus was first discovered and isolated in cells from African Burkitt's lymphoma by Epstein Barr and Achong in 1964 (Epstein, 1964), and have been reported to establish latent asymptomatic infection in about

90% of the world's adult human population (Shannon-Lowe *et al.*, 2017). Socioeconomic and developmental factors have been shown to influence the age at which primary infection can occur. For instance, in Sub-Saharan African countries where standard of living is poor, primary infection occurs in early childhood and most infected children seroconvert by the age of 3 years, whereas in developed or affluent countries, primary infection is delayed until late childhood or young adulthood (Rickinson, 2006).

To establish primary infection, the virus is transmitted through oral route where it exhibits dual tropism by infecting two main physiological targets, epithelial cells and B lymphocytes (Longnecker *et al.*, 2013). In addition to infecting the epithelia and B cells, the virus has also been shown to infect unnatural targets such as T lymphocytes and natural killer (NK) cells (Saha *et al.*, 2011). Interaction of EBV with epithelial cells remains inconclusive (Smatti *et al.*, 2018); one study reported that the virus infects epithelial cells by using viral envelop protein B cell maturation factor responsive 2 (BMFR2) to engage intergrins on the host cell surface (Tugizov *et al.*, 2003), however another study later reported that EBV uses glycoprotein gHgL to engage integrin αvβ5, αvβ6, or αvβ8 complex of the epithelia cell to trigger fusing and entering (Chesnokova *et al.*, 2014). An *in vitro* study also suggests that epithelial cells are infected through the transfer of EBV-coated B lymphocytes (Shannon-Lowe *et al.*, 2006), which was proven when epithelial cells were co-cultured with cell-free EBV preparation and low rate of infection was observed (Shannon-Lowe *et al.*, 2009).

Lytic replication of the virus occurs in the epithelial cells, but the virus can establish latency by infecting B cells found in the pharyngeal lymphoid tissues of the Waldeyer's ring (Shannon-Lowe *et al.*, 2017; Thorley-Lawson *et al.*, 2013). Upon entering the B cells, the viral genome either gets integrated into the host genome and persists as a provirus (Delecluse *et al.*, 1993) or remain in the nucleus as a non-integrated circular episome and expresses restricted set of genes that drive latency and survival of the host cell (Kang *et al.*, 2015; Yates *et al.*, 1984). The expression of the latency stage genes, known as latency programs, in the B cells lead to B cell-derived lymphomas as a result of the transformation of the cells into lymphoblastic lines (Table 1). The virus can be reactivated from latency in the B cells by a mechanism that is yet to be elucidated. In immunocompetent individuals, viral titres are held in check by EBV specific cytotoxic T cells (Hatton *et al.*, 2014). Although EBV undergoes lytic replication in the epithelia cells, where lytic stage genes are expressed, the virus can switch to the expression of latency stage genes, and lead to the transformation of the epithelia cells into permanently proliferating cells and resulting in epithelia cell derived malignancies (Table 1) (Tsao *et al.*, 2015).

Two general models; germinal center (GC) process in naïve cells and direct infection (DI) of memory cells, have been proposed to describe the persistance of EBV in B cells (Thorley-Lawson *et al.*, 2008). In the germinal center model, naïve B cells are infected and activated by the virus, leading to their proliferation into latently infected lymphoblasts. The virus then expresses limited sets of latent proteins, known as growth transcription programs (Latency III). The activated cells migrate to the follicle to start the GC reaction for the establishment of default transcription program (Latency II)(Hochberg *et al.*, 2004). This default program provides rescue or survival signal for the cells to exit the GC as memory B cells, followed by the complete silencing of viral protein expression in these cells to evade immune recognition (Latency 0). However, the virus switches to Latency 1 during occasional division of the B cells, to ensure the segregation of the viral episome into daughter cells. The memory cells may return to the tonsil and differentiate to plasma cells which activates viral replication and the release of the virus into the saliva for transmission (Young *et al.*, 2004). For the direct infection (DI) model, proposed by Rajewsky and colleagues (Kurth *et al.*, 2003), memory B cells are infected directly by the EBV and these cells are driven to proliferate without going through the GC reaction (Chaganti *et al.*, 2008). However, this model has been disproved by investigators because it has no mechanistic evidence backing it (Hawkins *et al.*, 2013). When this model was experimentally tested, the findings obtained were inconsistent and rather supported the GC model. For instance, in vivo studies have shown that viral default transcription program (Latency 2) was expressed in infected GC B cells and this finding was consistent with the GC model, but conflicted with the DI model which predicted Latency 3 (Babcock *et al.*, 2000; Roughan *et al.*, 2009).

The genome of EBV is a linear double-stranded DNA of approximately 172-kbp, and has several open reading frames which comprises of genes for the latent and lytic stages (Ai *et al.*, 2012). The latent stage genes include Epstein Barr nuclear antigens (EBNA), latent membrane protein 1 (LMP1), and Epstein Barr encoded RNA (EBER), while the lytic stage genes are BamHI left frame 1 (BZLF1) and viral capsid antigens. The virus is categorized into two major types (types 1 and 2) based on significant allelic polymorphisms within the EBV nuclear antigen 2 gene (EBNA2) (Sample *et al.*, 1990). Type 1 EBV is found predominantly in Europe, America, China and South-East Asia, with minority of the populace in these regions having type 2 EBV. However, in Africa and Papua New Guinea type 2 EBV is the most common but some people harbor both viral types, especially in immuno-compromised individuals (Aitken *et al.*, 1994). The two viral types are further classified into seven strains based on genetic variations such as the presence or absence of a unique 30-bp sequence at the C-terminal region of the LMP1 gene (Edwards *et al.*, 1999). China1, China 2, China 3, Alaskan (AL), Mediterranean with (Med+) or without (Med-) deletions, and North Carolina (NC) are the commonly reported strains based on LMP1 polymorphism, which has been implicated in tumor formation (Ai *et al.*, 2012). The EBV strains with deleted 30-bp at the carboxyl terminal region of LMP1 gene have been shown to be implicated in nasopharyngeal cancers (NPC) and other malignancies such as Burkitt's lymphoma, Hodgkin lymphoma, posttransplant lymphoproliferative disorders, NK/T cell lymphoma, gastric cancer, and breast cancer (Corvalan *et al.*, 2006; Deyrup, 2008). The oncogenic ability of LMP1 was proven by Moorthy *et al*. (1993), where LMP1 was shown to transform rodent fibroblast *in vitro* to cause tumor (Moorthy *et al.*, 1993). Some of the EBV strains have also been reported to present in the peripheral blood mononuclear cells as well as the saliva of infected individuals (Srivastava *et al.*, 2000). Since a majority of the world's population are asymptomatic carriers of EBV (Calderwood *et al.*, 2007) and most of the infections have been associated with the incidence of NPC in both endemic and non-endemic regions, there is the possibility of the involvement of EBV subtypes in the etiology of NPC cases in Ghana. Although, many studies have been done on the association of EBV types and strains and their tumorigenic potential in nasopharyngeal carcinogenesis in endemic areas such as South-East Asia, few studies have been done on the EBV genotypes involved in the etiology of NPC cases in non-endemic regions in Africa (Busson *et al.*, 2004). Against this backdrop, the study will be the first to assess the genotypes of EBV implicated in NPC cases in Ghana.

## 1.2 Problem Statement

Nasopharyngeal cancer incidence shows remarkably different geographical distribution, which is inconsistent in comparison to the widespread infection of EBV. The virus has been implicated in the etiology of NPC due to the expression of latent phase gene LMP1 which causes the transformation of B cells into permanently growing lymphoblastic cell lines (LCLs). Studies conducted in other countries reported genetic variations in EBV genotype and these variations are geographically specific, suggesting that there are disease-specific EBV genotypes preferentially virulent to specific populations, and making them more prone to NPC. The two genotypes also vary in biological properties; EBV genotype 1 is more efficient in immortalizing B cells and genotype 2 has a higher lytic ability. A previous study on NPC cases in Ghana (Kitcher *et al.*, 2004) did not take into account the main etiological agent, EBV, involved in the pathogenesis of the NPC. Hence, little is known about the circulating EBV genotypes that may be present in people with NPC in Ghana, there is therefore the need to identify and characterize EBV genotypes that may be present in Ghanaian patients and which may be involved in the pathogenesis of NPC.

## 1.3 Justification of Study

The diagnosis of NPC is mostly difficult due to non-specific clinical manifestations which results in the recognition of the disease only after tumor formation. The current treatments available, which include chemotherapy and radiotherapy, are expensive and can only be afforded by few people. Besides being expensive, radiotherapy could cause other cancers in patients. Coupled with the lack of adequate information on the disease specific EBV genotypes implicated in NPC cases in Ghana, the problem of the disease may even be more complicated. Also the identification of EBV DNA in the blood or biopsies does not predict or correlate with symptomatic infection in both immunocompetent and immunocompromised individuals. However, the viral burden/load has been associated to symptomatic infection and rapid indicator of immunopathological alterations fundamental to epithelia or B cell driven changes associated to EBV. Quantification of EBV DNA levels has proven useful in prognostication, early detection, tumor staging and monitoring of treatment response in NPC patients. The goal of this study is to determine the genotypes of EBV in blood and biopsies of NPC patients, and this will give a snapshot of genotype profiles in the various samples. This study is also aimed at quantifying the viral load in the various specimens and this will serve as prognostic marker for disease diagnosis, treatment and management.

### 1.4 Hypothesis

It is hypothesized in this study that EBV genotypes are responsible for the development of nasopharyngeal cancers in patients reporting to the Ear, Nose and Throat Unit (ENT) of Korle-Bu Teaching Hospital (KBTH) in Ghana.

### 1.5 Aim

The purpose of the study is to detect and characterize EBV genotypes associated with NPC cases in Ghana.

### 1.6 Specific Objectives:

1. To identify and characterize EBV genotypes 1 and 2 in NPC biopsies and whole blood from NPC patients
2. To determine EBV load in the various specimen using real-time polymerase chain reaction (PCR)

# CHAPTER TWO

## 2.0 Literature Review

### 2.1 Genome structure of EBV

The genome of EBV is a linear, double stranded DNA, approximately 172 Kbp with more than 85 protein coding genes (Open reading frames (ORF)) (Bouvard *et al.*, 2009; Santpere *et al.*, 2014). The ORFs encode proteins involved in regulation of DNA replication and gene expression, and maintenance of genome integrity in daughter cells (Böhm, 2010). The nomenclature of the ORFs was determined based on the *Bam* HI restriction fraction map in which the genes were in decreasing order according to their sizes (Corvalán *et al.*, 2019). The protein coding genes are divided into lytic and latent genes which play structural and non-structural roles. The viral genome is further divided into long and short unique sequence domains which are separated by a series of 0.5 Kbp terminal direct repeats that are found at the end of each sequence domain (Arvin *et al.*, 2007). The terminal direct repeats increase the coding capacity of the genome and also serve as a biomarker to determine the progenitor origin of EBV in the infected cells (Bouvard *et al.*, 2009; Smatti *et al.*, 2018).

### 2.2 Classification and Geographical Distribution of EBV

EBV can be classified into two main genotypes, 1 and 2, based on sequence divergence in the nuclear proteins, Epstein Barr Nuclear Antigen (EBNA)-1, -2, -3A, -3B, and 3C (Banko *et al.*, 2016). However, EBNA-2 is generally used for the classification because the protein has the least percentage of sequence homology between the two genotypes (Münz, 2015; J Sample *et al.*, 1990). The two EBV genotypes occur worldwide but vary in geographical distribution; EBV genotype 1 is found globally but predominant in American, Chinese, European and South-East Asian (SEA) populations whereas genotype 2 is predominantly found in Africa (Peh *et al.*, 2002). The two genotypes also vary in biological properties; EBV genotype 1 is more efficient in immortalizing B cells and the genotype 2 has a higher lytic ability (Griffiths, 2004; Klemenc *et al.*, 2006). The virus is further classified into seven different strains: China 1 (C1), China 2 (C2), China 3 (C3), Mediterranean with (Med+) or without (Med-) deletions, Alaskan (AL), and North Carolina, based on nucleotide polymorphism, 30 bp deletions, or signature amino acid changes in the LMP1 gene as compared to the sequence of a prototype EBV, B95-8 (Banko *et al.*, 2016; Miller *et al.*, 1994). The different strains show diversity in tumorigenic activity; a 30 bp deletion at the carboxyl terminal, for example, has a higher tumorigenic activity and poor immunogenicity compared to the undeleted variant (da Costa *et al.*, 2015).

## 2.3 Biological Activities of EBV-Encoded Latency Associated Gene Products

Establishment of latent infection by EBV has been implicated in several malignancies (See *et al.*, 2008) due to the expression of limited sets of latent proteins, shown to play various biological roles discussed below (Table 1.1).

**Table 1.1: Biological activities of Epstein Barr virus latency proteins and associated cancers**

| EBV latency protein | Type of latency | Biological activity | Associated cancers[d] |
|---|---|---|---|
| **EBNA-1**[a] | Latency I, II, III | Segregation of viral genome progenies, DNA replication, inhibition of MHC class I, enhances p53 degradation | Burkitt lymphoma, Gastric cancer,<br>Breast cancer |
| **EBNA-2** | Latency III | Upregulation of host and viral proteins (transactivation), facilitate B cell immortalization | Posttransplant lymphoproliferative disorder |
| **EBNA-3** | Latency III | Transcription transactivation of both host and viral proteins, immortalization of B cell | Posttransplant lymphoproliferative disorder |
| **EBNA-LP**[b] | Latency III | Transactivation of EBNA-2 to inactivate tumor suppressors, essential for immortalization of B cells | Posttransplant lymphoproliferative disorder |
| **LMP-1/2**[c] | Latency II/III | B cell survival, upregulation of antiapoptotic proteins, mimics CD 40 ligand associated signaling, constitutively activate growth and cell survival promoting signaling pathways | Hodgkin lymphoma,<br>Nasopharyngeal cancer<br>Posttransplant lymphoproliferative disorder<br>T/NK cell lymphoma<br>Breast cancer |

**[a] EBNA-1 is expressed and detected in all EBV associated malignancies**
**[b] EBNA-LP is also known as EBNA-5**
**[c] LMP-1/2 are both involved in epithelia and B cell tumors, however, LMP 2 is frequently detected in a majority of all tumors as compared to LMP-1.**
**[d] The associated tumors are not only limited to the ones discussed in this review**

### 2.3.1 Epstein Barr Nuclear Antigens (EBNAs)

Six EBNAs (1, 2, 3A, 3B, 3C and LP) are expressed during latent infection in host cells, and their biological functions have been reported by researchers worldwide (AlQarni *et al.*, 2018; Kieff, 2007). The expression of these antigens vary across the latency programs establish by EBV in infected cells. During latency III, all the six EBNAs are expressed, however in latency II and I, only one EBNA (EBNA-1) is expressed, but no EBNA protein is expressed during latency 0 (Kieff, 2007; Rickinson, 2006). All the EBNAs are involved in regulation of transcription and also participate in transcriptional activation of viral LMP genes (*LMP*-1 and -2) in infected cells (Rowe, 1999; Thompson *et al.*, 2004).

EBNA-1 is a sequence specific DNA-binding protein that interacts with three unique palindromic sequence target sites (family of repeat elements (FR), dyad symmetry element (DS), and sequences found downstream of the Q promoter (Qp)), which are repeated multiple times on the viral genome (Dresang *et al.*, 2009). EBNA-1 binds to FR elements, which act as enhancer for viral C promoter, to direct the transcription of all the six EBNAs. Whereas engaging the DS elements by EBNA-1 leads to regulation of S-phase associated viral DNA replication, interaction with Qp down-regulates the transcription of Qp-driven EBNA-1 (Frappier *et al.*, 1994; Sample *et al.*, 1992). EBNA-1 also ensures the segregation of the viral genome in daughter cells and up-regulates LMP promoter, sustaining cell survival or immortalization of infected cells (Kieff, 2007). A more recent study has reported that EBNA-1 derived from nasopharyngeal cancer is required for the maintenance of EBV episome and DNA replication during latent infection (Dheekollu *et al.*, 2017). EBNA-1 protein is made up of N and C termini which are separated from each other by variable repeats of glycine-alanine sequences. The amino acid sequence repeats hide EBNA-1 from immune recognition by a cis-acting inhibitor of major histocompatibility complex (MHC) class I, and prevent antigen presentation through ubiquitin-proteosome pathway (Levitskaya *et al.*, 1995; Levitskaya *et al.*, 1997).

Unlike EBNA-1 which binds to DNA, EBNA-2 does not interact with DNA but engages cellular transcription factors such as CBF1/RBP-Jk, PU.1, and other proteins to up-regulate the expression of both viral and cellular genes (Kaiser *et al.*, 1999). The cellular genes that are up-regulated by EBNA-2 include CD 23 and *c-myc* found in B cells. Activation of *c-myc* leads to expression of proteins such as D-type cyclins and cyclin E, which are associated with cell division (Wood *et al.*, 2016). EBNA-2 also interacts with other transcription factors such as Cp, LMP-1, and -2 binding factors for up-regulation of viral genes that are responsible for immortalization of B cells (Kaiser *et al.*, 1999; Klein, 2007; Radkov *et al.*, 1997).

EBNA-3 family of nuclear proteins consist of three large nuclear phosphoproteins, EBNA-3A, -3B, and -3C (Klein, 2007). All the EBNA-3 proteins share few sequence homology at the N terminus region, with the conserved domain used to engage cellular transcription factors such as CBF1/RBP-Jk (on the Evaluation, 2012). By binding to CBF1/RBP-Jk, EBNA-3 acts as a repressor of EBNA-2-mediated transactivation of cellular and viral proteins (Wang *et al.*, 2016). *In vitro* genetic studies have shown that only EBNA-3A and -3C are required for immortalization of EBV-infected cells (Maruo *et al.*, 2011; Saha *et al.*, 2013). Although EBNA-3 B is dispensable, a report has shown that the protein regulates B cell homing by altering the expression of chemokine receptor 4 (CXCR4) (Chen *et al.*, 2006).

Epstein Barr nuclear antigen leader protein (EBNA-LP) also known as EBNA-5 is a nuclear phosphoprotein that is co-expressed with EBNA-2 upon EBV infection of target cells (Alfieri *et al.*, 1991). The co-expression of EBNA-LP and EBNA-2 has been shown to enhance EBNA-2 transcriptional activation of both cellular and viral proteins (Harada *et al.*, 1997; Klein, 2007).

EBNA-LP plays a key role in B cell transformation into a permanently proliferating lymphoblast based on the observation that cells expressing the mutant genes were less transformed as compared to cells with wild type EBNA-LP gene (Allan *et al.*, 1992; Mannick *et al.*, 1991). The leader protein interacts with cellular proteins, some of which are oncogenic and tumor suppressors (pRb, p53, p14ARF, and Fte1/S3a), cell cycle regulators (DNA-PKcs and HA95), and an anti-apoptotic protein (HAX-1) (Dufva *et al.*, 2001; Han *et al.*, 2001; Kashuba *et al.*, 2005). EBNA-LP up-regulates the expression of thymus and activation regulated chemokine (TARC) gene, which has been proposed to play a key role in B cell transformation and survival (Imai *et al.*, 1999; Kanamori *et al.*, 2004). Previous studies have reported that EBNA-LP stimulates EBNA-2 transactivation of viral proteins such as latent membrane proteins 1 and -2B (LMP-1 and -2B) in immortalized cells (Harada *et al.*, 1997; Nitsche *et al.*, 1997; Peng *et al.*, 2004).

### 2.3.2 Latent Membrane Protein (LMP)

Three latent membrane proteins, LMP-1,-2A, and -2B, are expressed by EBV during latency II and III in EBV infected cells (Webster-Cyriaque *et al.*, 1998). All the three proteins are expressed as cell surface membrane proteins, and are required for survival and transformation of the infected cells into permanently proliferating cells.

Latent membrane protein-1(LMP-1) gene is an essential oncogene, which is expressed as a constitutively active receptor in a majority of EBV associated tumor cells (Siegler *et al.*, 2003). Latent membrane protein-1 is a 356-amino acid protein with a short cytoplasmic N-terminal domain, six trans-membrane spanning domain, and a 200 amino acid long C-terminal cytoplasmic domain (Liebowitz *et al.*, 1986). The N-terminal domain tethers the LMP-1 on the surface of the plasma membrane, and oligomerization as well as self-aggregation of the protein is mediated by the six trans-membrane spanning domain. The C-terminal is the biologically active region of LMP-

1, with two functional domains, namely, C-terminal activating regions (CTAR)-1 and -2, which are needed for efficient EBV-mediated B cell transformation (Huen *et al.*, 1995). Previous reports have shown that these two functional domains interact with intracellular signaling proteins such as tumor necrosis factor (TNF) receptor-associated factor (TRAF)-1, -2, -3, -4,-5, and -6 found in tumor necrosis factor receptor (TNFR) signaling pathway in B cells and epithelia cells (Arcipowski *et al.*, 2011; P. Xie *et al.*, 2004). By interacting with these adaptor molecules, CTAR constitutively activates major signaling pathways such as the extracellular signal-regulated kinase (ERK), mitogen-activated protein kinases (MAPKs), PI3K/Akt, AP-1, Jun N-terminal protein kinase (JNK), the p38 and canonical and non-canonical NF-κB, and JAK/STAT pathways (Lam *et al.*, 2003; Morris *et al.*, 2009; Tsao *et al.*, 2002). The activation of these major signaling pathways results in the up-regulation of expression of antiapoptotic proteins such as A 20, Bcl-2, and also down-regulation of tumor suppressor protein, p53, promoting cell growth and survival (Hatzivassiliou *et al.*, 2002; Tao *et al.*, 2015). A more recent study has shown that the activation of AP-1, JAK/STAT, and NF-κB pathways by CTAR resulted in increased expression of programmed cell death protein 1 ligand (PD-L1), a key immune checkpoint suppressor in tumor immunology (Fang *et al.*, 2014).

Latent membrane protein-2 (LMP-2) is made of two variants, LMP-2A and -2B, which are expressed during latency II and III in EBV infected cells (Rickinson, 2006). Structurally, both proteins have 12 trans-membrane domains and a 27 amino acid cytoplasmic C-terminal domain, however, LMP 2A has an additional 119 amino acid cytoplasmic N-terminal domain, which is not found in LMP 2B (Rovedo *et al.*, 2007). The N-terminal cytoplasmic domain of LMP 2A contains immunoreceptor tyrosine-based activation motif (ITAM), which mediate B cell receptor (BCR)-like signaling cascade in EBV infected cells (Beaufils *et al.*, 1993; Fruehling *et al.*, 1997). The

BCR-like signaling, mediated by LMP2A, provides strong survival signal which rescues BCR-negative cells from apoptosis (Caldwell *et al.*, 1998; Merchant *et al.*, 2000) and inhibits signaling that leads to lytic reactivation ( Miller *et al.*, 1994). LMP2A has been shown to be essential for activation, proliferation, and survival of EBV infected B cells at early times, after which it is required for long term growth of B cells (Wasil *et al.*, 2013). Further study has shown that LMP2A helps EBV infected cells to escape elimination by CD8$^+$ T cells by down regulating (i) the expression of CD8$^+$ T cells specific latent stage genes, especially EBNA-1, (ii) the expression of MHC class 1 proteins to prevent antigen presentation, and (iii) the expression of coactivatory receptor NKG2D to evade T cell recognition (Rancan *et al.*, 2015). The function of LMP 2B on the other hand, is not clearly known (Wasil *et al.*, 2013), but previous report have shown that it interacts with LMP 2A to modulate the activities of the latter (Rovedo *et al.*, 2007).

### 2.3.3 EBV-MicroRNAs (EBV-miRNA)

MicroRNAs (miRNA) are small non-coding RNA molecules, usually 18-24 nucleotides in length, which transcriptionally down-regulate expression of complementary mRNAs (Dong *et al.*, 2019). miRNAs have been identified in different organisms such as mammals, plants, algae and worms, however, EBV was one of the first viruses identified to express these small non-coding RNA molecules (Alptekin *et al.*, 2017; Pfeffer *et al.*, 2004). The virus has been found to express about 44 miRNAs in all forms of latency and tumor tissues, regulating both cellular and viral genes (Shinozaki-UshikuKunita *et al.*, 2015b). These 44 miRNAs are produced from two miRNA coding regions: the BamHI fragment H rightward open reading frame 1 (BHRF1) region which encoded 4 miRNAs, and the BamHI A rightward transcript (BART) region which encodes the remaining 40 miRNAs (Barth *et al.*, 2011). Since EBV miRNAs are detected in EBV associated tumors, it is believed that they play important roles in the pathobiology of the life cycle of EBV and tumors associated to EBV (Navari *et al.*, 2018).

EBV establishes latency, where it persists for life in the host by developing complex mechanisms for evading immune recognition. The miRNAs help the virus to escape immune surveillance by down-regulating the expression of both immunogenic viral antigens and host immune proteins (Dong *et al.*, 2019). For instance, Lung and colleagues in 2009 reported that BART 22, encoded by the BART miRNA region, down-regulated the expression of LMP 2A, an immunogenic viral protein, and enhances NPC tumorigenesis (Lung *et al.*, 2009). Also, Impotin 7 (IPO7) which is a cellular receptor responsible for transporting transcription factors into the nucleus and plays a key role in innate immunity, has been shown to be inhibited by EBV miR-BART3 and miR-BART16 (Dölken *et al.*, 2010; Yang *et al.*, 2009). A previous study has demonstrated that miR-BART2-5p protects EBV-infected cells from immune recognition by natural killer (NK) cells through the inhibition of major histocompatibility complex (MHC) class I chain-related molecule B (MICB) which engages receptor natural killer group 2D (NKG2D) of the NK cells (Bauer *et al.*, 1999; Nachmani *et al.*, 2009). EBV miRNA BART 16, has been shown to disrupt Type I IFN signaling pathway by down-regulating the expression of CREB-binding protein, a transcriptional activator of IFN, hence suppressing host immune response against the virus (Hooykaas *et al.*, 2017). EBV also produces BART6-3p which interacts with miR 97, a cellular microRNA, to synergistically suppress the expressing of Interleukin-6 receptor (IL-6R) which is a receptor for several antiviral cytokines such as IFN-α, IL-12 and IL-27 (Y. M. Zhang *et al.*, 2017), promoting tumor progression in both epithelia cancers and lymphomas.

Aside targeting cellular cytokines, EBV miRNAs, inhibit the destruction of B cell and epithelial cell derived cancers by down-regulating expression of tumor suppressor genes. miR-BHRF1-2 has been reported to silence the expression of PRDM1/Blimp1which is a tumor suppressor in B cells, hence promoting lymphogenesis (J. Ma *et al.*, 2016). The virus also targets the mitogen-activated protein kinase (MAPK) signaling pathway which suppresses tumor formation, by producing BART 22, shown to inhibit MAP kinase kinase kinase 5 (MAP3K5) expression (R. Chen *et al.*, 2017).

## 2.4 Detection of EBV

Epstein Barr virus is implicated in several malignancies ranging from epithelia and lymphoid origins. Biochemical, serological, and molecular methods to detect EBV are increasingly necessary in diagnosis and monitoring of patients with EBV implicated diseases (Gulley, 2001). Some of the techniques used to detect the virus include: serological assay, immunohistochemistry, EBER *in situ* hybridization, viral detection and viral load quantification using Real-time PCR, and EBV clonality assay using Southern blot analysis.

**Serological assay**: Serological detection of antibodies against EBV specific antigens such as viral coat antigen (VCA), EBNA-1, early antigen (EA) and viral nucleic acids, have been widely used to screen and monitor EBV infections (Chen *et al.*, 2015; Gao *et al.*, 2017; Shao *et al.*, 2004). Enzyme linked immunosorbent assay (ELISA) and immunofluorescent assays have been the most common platforms used to detect EBV-specific antibodies. The type of antibody profile generated from these platforms can be used to classify the infection as acute, primary and past infections (Nystad *et al.*, 2007). The first antibody to be produced during acute primary infection, is IgM against VCA (IgM-VCA). IgG antibodies (IgG-VCA) may be produced but at a level below detection. However, between 2 to 6 months, IgM-VCA levels decline and IgG-VCA levels peak and sustained for life long. Also IgG antibodies against EBNA-1, IgG-EBNA-1 peak up between the third and sixth months are maintained for life and can serve as markers for prior infection or reactivation of EBV(Odumade *et al.*, 2011).

**Immunohistochemistry**: Immunohistochemistry (IHC) is a technique used to detect antigens in tissues based on chemical and immunological reactions (Ramos-Vara, 2011). This method has been employed to stain and detect EBV proteins such as LMP 1, LMP 2A, EBNA-1, -2, and BZLF1, in paraffin section (G. Niedobitek *et al.*, 1997; Pinkus *et al.*, 1994; Yates *et al.*, 2017). IHC is further used to confirm the diagnosis of EBV implicated disease. For instance, hairy leukoplakia, an EBV implicated disease in AIDs patients is confirmed by immunostaining of BZLFI in tongue biopsies (Cruchley *et al.*, 1997).

**EBER *in situ* hybridization**: Epstein Barr encoded RNA (EBER) *in situ* hybridization (EISH) has been considered the gold standard for detection and diagnosis of EBV infection (Richard F Ambinder *et al.*, 1994). EBER is a small non coding and non-polyadenylated RNA which is highly expressed in all EBV latently infected cells, irrespective of cell phenotype (Samanta *et al.*, 2008). Two types of EBERs are transcribed by EBV during latent infection, namely EBER-1 and -2, which are 166 and 172 nucleotide long, respectively (Clemens, 1993). Because EBER is expressed in high copies, it serves as reliable target for the detection of EBV infected cells. The EISH technique employ the use of nucleic acid probes, either label or unlabeled, which can hybridize with EBER on paraffin sections of EBV infected tissues (Cao *et al.*, 2017).

**Viral detection and viral load quantification using Real-time PCR**: Detection and quantification of EBV nucleic acids using polymerase chain reaction (PCR), in body fluids and tissues has been shown to be a powerful tool for diagnosing and monitoring of EBV associated diseases(Gulley *et al.*, 2010). Technically, to amplify EBV DNA, primers are designed to span conserved regions in the EBV genome. This method has been used to detect the presence of EBV in peripheral blood mononuclear cells, plasma, saliva, tumor biopsies and cerebrospinal fluid (CSF) (Arribas *et al.*, 1995; Klemenc *et al.*, 2006). The detection of EBV in these various samples has prognostic implications. For example, the detection of EBV DNA from CSF of patients with HIV/AIDS is an indication of brain lymphoma and this compels oncologist to administer lymphoma treatments without taking brain biopsies (Antinori *et al.*, 1999). Although, PCR amplification of EBV DNA in infected cells is very sensitive, it fails to distinguish EBV disease from background infection, since about 90 % of the world's population harbors this virus (Gulley, 2001). To avoid the risk of false positive, many laboratory scientists prefer to use EISH assay for the reliable detection of EBV in lesions. It has been reported that quantitative viral load assessment using real-time PCR (QPCR) is superior to conventional PCR which is qualitative (Fafi-Kremer *et al.*, 2005). QPCR assay involves the multiplex amplification of a target EBV gene and internal control nucleic acid from blood samples. The amount of amplicons measured at the end point of the assay or in real time, can be used to calculate the EBV load in copies per milliliter of blood, for prognostic inferences to be made (Killeen, 2000). There are no established threshold levels that should inform a change in therapeutic strategy. Although, the WHO in 2011 developed and approved EBV international standard (EBV IS) for quantification of EBV load, variation still exists in reported values across different laboratories (Fryer *et al.*, 2011). This is attributed to the fact that the gene targets, sample types, and assay platforms vary across different laboratories (Smith *et al.*, 2007). Moreover, there is still a debate on the best matrix for monitoring EBV infection. Some laboratory scientists prefer plasma to whole blood, whereas majority of experts recommend the use of whole blood (Rowe *et al.*, 2001; Stevens *et al.*, 2001; Wagner *et al.*, 2001). The viral load in plasma samples is about 10 to 100 fold lower than that of whole blood sample (Odumade *et al.*, 2011). Like conventional PCR, QPCR assay is not able to distinguish between

EBV DNA located in normal tissues and those coming from EBV lesions, this makes EISH remain as the gold standard for EBV detection (Ambinder *et al.*, 2005).

**EBV clonality Assay**: The clonality of EBV infected cells can be detected by southern blot analysis of unique EBV DNA sequences found in these infected cells. EBV genome contains variable numbers of terminal repeats flanking the ends of each EBV DNA molecule, making the DNA to form circular episome after infection (Banati *et al.*, 2017). These sequences serve as biomarkers for determining whether a given EBV infected cells originates from the same progenitor or not. The detection of the same number of terminal repeats in EBV infected cells could infer that the cell originates from the same progenitor, indicating latent infection. However, cells with different number of terminal repeats indicates polyclonal origins and the type of infection is lytic (Raab-Traub *et al.*, 1986). For example, by using southern blot analysis, it has been reported that EBV genome from oral hairy leukemia cells are polyclonal, suggesting lytic stage infection. However, monoclonal EBV DNA are found in EBV-associated tumors, indicating latent stage infection (Gulley *et al.*, 1996; Raab-Traub *et al.*, 1986).

### 2.5 Malignancies Caused By EBV Infection

Epstein Barr virus is linked to the pathogenesis of lymphomas such as Burkitt’s lymphoma (BL), Hodgkin lymphoma (HL) and post-transplant lympho-proliferative disorder (PTLD). Epithelial malignancies that are associated with EBV in epithelial cells include nasopharyngeal cancer (NPC), gastric cancer (GC) and breast cancer (BC) (Thompson *et al.*, 2004). In rare cases, EBV has been shown to infect NK and T cells; causing extra-nodal nasal-type NK/T cell lymphoma (Fox *et al.*, 2011). The different malignancies are shown in Figure 1.1.

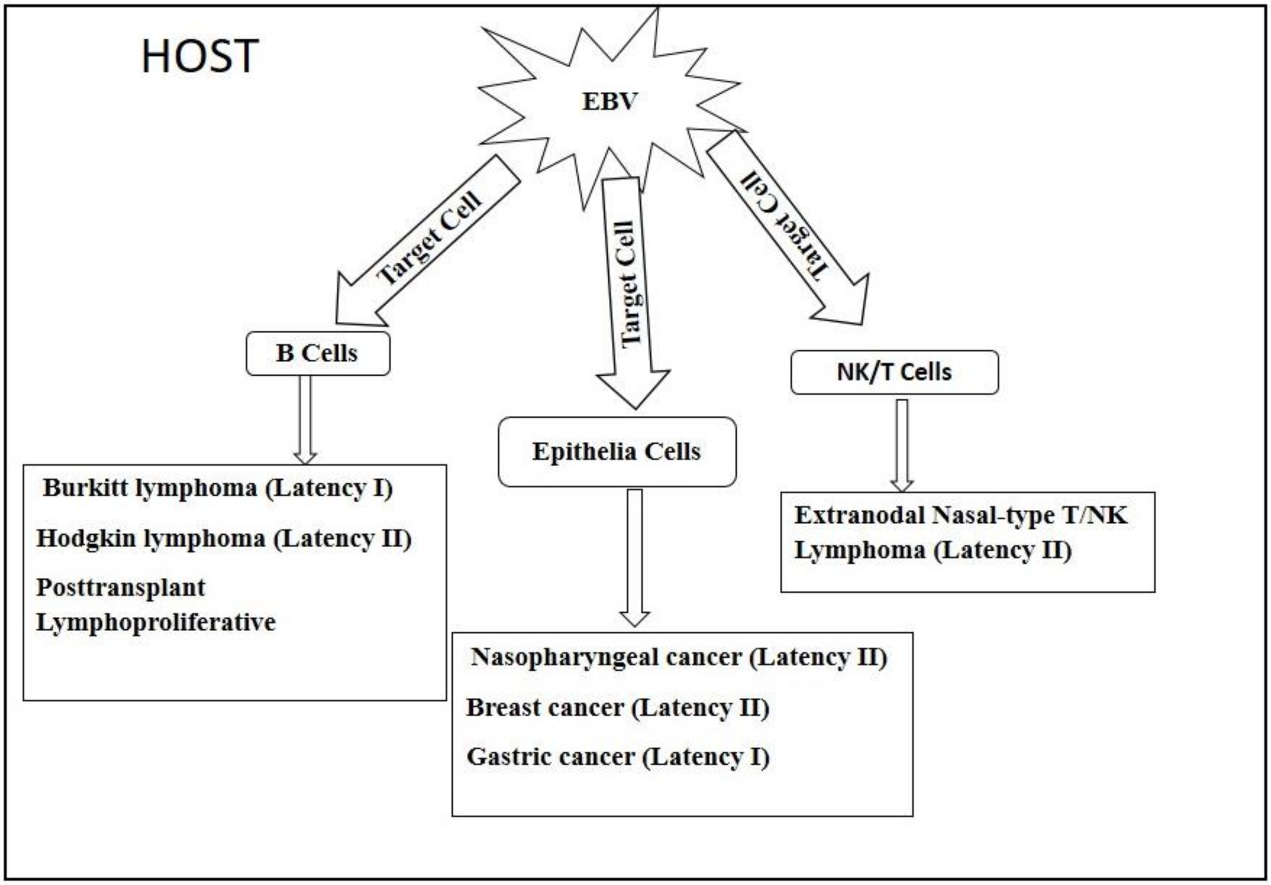

**Figure 1. 2 Diagrammatic view of EBV associated epithelia and hematopoietic cell derived malignancies.** Upon entering into the host, EBV has tropism for two main targets, namely B cell and epithelial cells as well as unnatural targets such as NK/T cells. The virus in the various target cells expresses different patterns of latency genes and these determines the type of cancer that would be developed. B cell derived lymphomas include Burkitt lymphoma, Hodgkin lymphoma, and posttransplant lymphoproliferative disorders which are caused by the expression of latency I, II, and III, respectively, gene products. Infection of epithelial cells by EBV results in cancers such as nasopharyngeal, breast, and gastric cancers, caused by the expression of latency II, II, I gene products, respectively. The virus can also invade unusual targets such as natural killer (NK) cells and T lymphocytes to cause extranodal nasal-type T/NK lymphoma due to the expression of latency II gene products. Full details of each tumor are further described below.

### 2.5.1 Burkitt’s Lymphoma (BL)

Burkitt’s lymphoma (BL) is a highly aggressive non-Hodgkin B cell neoplasm, and is the world’s commonest pediatric cancer endemic in Sub-Saharan Africa (Rochford *et al.*, 2015). The pathogenesis of BL has been linked to EBV infection as a result of the isolation of the virus from cultured BL cell lines in 1964 (Epstein, 1964). The virus has since been implicated in 95% of BL cases from high risk regions and less than 30% from low risk regions (Magrath, 1991). BL occurs in children living in areas that are both holo-endemic and hyper-endemic for malaria, suggesting that *Plasmodium falciparum* plays a role in the etiology of the cancer (Thorley-Lawson *et al.*, 2016). Burkitt’s lymphoma is classified into three different forms based on clinical observations and disease epidemiology (Swerdlow *et al.*, 2016). Endemic BL (eBL) has an annual incidence of about 5-10 cases per 100 000, and contributes to 50% of all pediatric cancers in malaria endemic regions such as Equatorial Africa and Papua New Guinea, where EBV is found in about 95% of all diagnosed cases (Bornkamm, 2009; Lenze *et al.*, 2011). Common sites of tumor occurrence in eBL are the jaws and abdomen (Aguilar *et al.*, 2017). Sporadic Burkitt’s lymphoma (sBL), on the other hand, has a wide global distribution, but is at a much lower frequency and mostly diagnosed in both children and young adults. In USA and Western Europe, sBL is responsible for 30-50% of all pediatric neoplasms and less than 1% in adults (Blum *et al.*, 2004). Epstein Barr virus is rarely linked to sBL cases diagnosed in the western world, however in other places like North East Brazil, the frequency of EBV in sBL exceeds 80% (Queiroga *et al.*, 2008; Shannon-Lowe *et al.*, 2017). In contrast, immunodeficiency-associated Burkitt’s lymphoma (iBL) has been found in HIV carriers who develop the lymphoma before progressing to AIDS. The incidence of iBL is 10 to 100-fold higher than the sporadic form of the disease, and about 30-40% of iBL are positive for EBV (Gloghini *et al.*, 2013). Immune compromise, which occurs as a result of HIV infection, accounts for reactivation of EBV from latently infected B cells and eventually causing rapid progression to iBL (Amria *et al.*, 2008). The CD4+ T cells prime CD8+ T cells against circulating EBV in immunocompetent individuals, and thereby bringing the virus under immune surveillance. However in HIV infected individuals the CD4+ T cell count is drastically reduced, and therefore reduces the priming of the EBV specific CD8+ T cells in these individuals (Pietersma *et al.*, 2008; Sebelin-Wulf *et al.*, 2007).

The expression of EBV genes in BL is strictly latent and restricted to the type I latency default programs. Only EBNA-1 and EBER are expressed in EBV positive cells in BL tumors (Tsao *et al.*, 2017). Whiles some studies have reported the detection of LMP1 and EBNA-2 in rare cases of eBL and LMP1 in many sBL cancers, these detections were not observed in all occurrences (Carbone *et al.*, 1996; Kelly *et al.*, 2006; G Niedobitek *et al.*, 1995). Even though the role of EBV in the pathogenesis of BL is still unclear, evidence have shown that the expression of EBNA-1 in BL cell lines promotes cell proliferation by inhibiting apoptosis (Kirchmaier *et al.*, 1997). Recent reports suggest that EBNA-1 inhibits apoptosis in BL cell lines by interacting with host proteins such as survivin, an anti-apoptotic protein and p53-regulator USP7 (Dheekollu *et al.*, 2017; Frappier, 2012).

All BL tumors, regardless of variants or EBV status, are morphologically and immunophenotypically identical and are related by sharing a general gene expression patterns which resemble that of centroblasts (Dave *et al.*, 2006; Hummel *et al.*, 2006). The BLs, in general, undergo chromosomal translocation of *c-myc* proto-oncogene on chromosome 8 or on chromosome 14 to one of the three immunoglobin loci, notably the heavy chain immunoglobin (Ig) (Guikema *et al.*, 2008). This brings the *c-myc* under the control of highly active Ig gene promoter leading to constitutive expression of *c-myc* proteins at high levels in BL cells. The expression results in uncontrolled cell growth in BL (Allday, 2009).

### 2.5.2 Hodgking Lymphoma (HL)

Hodgking Lymphoma is a lymphoid neoplasm which originates from B cell, characterized by the presence of few multinucleated giant cells known as Hodgkin/Reed Sternberg (HRS) cells and are surrounded by a non-neoplastic inflammatory infiltrate (Mirsadraee *et al.*, 2010). Hodgkin/Reed Sternberg cells are found in about 40-85% of all HL cases diagnosed in children below age 10 and 45-80% of cases reported in patients above 75 years (Glaser *et al.*, 1997). Hodgking Lymphoma incidence shows variation in geographical distribution, sex, ethnicity, and socioeconomic status. In more developed countries like the United States, the incidence of HL is higher and mostly among males, and less common in Asian countries (Mozaheb *et al.*, 2011). However, epidemiological report by Salati and colleagues has suggested that there is an inverse correlation between HL incidence and mortality in developed and under-developed countries. This observation was ascribed to improved health facilities that are mostly found in more developed countries as compared to the under-developed ones (Salati *et al.*, 2014).

Epstein Barr virus have been implicated in the pathogenesis of about 40% of all HL cases due to the detection of EBV DNA, EBER RNA , EBNA and LMP in HRS cells, establishing a link between EBV and HL (Kapatai *et al.*, 2007; Wu *et al.*, 1990). However, the rate of EBV-HL positivity differs globally, notably with respect to geography. It has been reported that 30-40% of HL cases in North America and Europe were mostly EBV positive, while in other parts of the globe such as Latin America, Asia and Africa, EBV was found in almost 100% of all HL reported cases (Glaser *et al.*, 1997). Despite the association of EBV to HL cases, the role of EBV in the pathogenesis of this malignancy still remains unknown (Massini *et al.*, 2009). Some researchers have proposed that the expression of LMP-1 is essential for anti-apoptosis since it activates growth promoting signaling pathways such as JAK/STAT, PI3K and NF-κB pathways, and also induces transcriptional changes in GC B cells that mark HRS cells (Vockerodt *et al.*, 2008).

Hodgking Lymphoma can be classified into two variants based on clinical and morphological differences (Vockerodt *et al.*, 2014). The first variant, known as nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), is rarely EBV associated (Wang *et al.*, 2014), hence would not be discussed in this review. The second variant known as classical Hodgkin lymphoma (CHL) has been reported to account for about 95% of all HL cases and is mostly linked to EBV (Swerdlow, 2008). Association between EBV and CHL has been suggested due elevated antibody titres against EBV latent antigens, which proceeded the development of the lymphoma after several years (Levine *et al.*, 1971). Also, the detection of EBV latent stage genes and RNA from the biopsies of CHL patients further confirmed the link between the disease and EBV (Wu *et al.*, 1990). CHL can further be divided into four histological subtypes, namely, mixed cellularity (MC), nodular sclerosis (NS), lymphocyte-rich (LR) , and lymphocyte depleted (LD) (Swerdlow, 2008). About 96% of all MC cases are EBV associated, whereas NS is less frequently linked to EBV infection (Pallesen *et al.*, 1991). On the other hand, LR is never EBV associated, with LD being rarely EBV implicated (Čičkušić *et al.*, 2007). One unique feature of all CHL subtypes is that the HRS cells are responsible for only 1-2% of the tumor mass (Mathas *et al.*, 2016).

### 2.5.3 Post-Transplant Lymphoproliferative Disorders (PTLDs)

Post-transplant lymphoproliferative disorder (PTLD) is a type of malignancy with life-threatening complications in transplant recipients of both solid organ and hematopoietic stem cell allografts (Rasche *et al.*, 2014). The lymphoma occurs as a result of increase proliferation of B cells after transplant, and it is mostly EBV driven (Kim *et al.*, 2017). The genome of the virus has been found in more than 90% of B cells from PTLD patients, either after primary infection, or reactivation of EBV from latently infected cells after treatment with immunosuppressant to avoid allograft rejection (Green, 2001). The use of immune suppressive drugs after organ transplant reduces the number and function of T cell to influence the success of the transplant. The use of the immunosuppressant leads to depletion of EBV-specific T cells and disruption of the balance between the immune system and latent virus, and consequently, there is reactivation of EBV from latency (Nijland *et al.*, 2016). The impairment of EBV-specific T cell mediated immune surveillance results in uncontrolled lymphoproliferative blast, which eventually causes PTLD in transplant recipients (Snow *et al.*, 2007; Stojanova *et al.*, 2011).

The prevalence of EBV- associated PTLD ranges from 1-20%, with incidence varying according to the type of allograft, age, and pretransplant EBV-serostatus of transplant recipient. Post-transplant lymphoproliferative disorder cases are highest (32%) in small intestine transplant recipients, and 3-12% incidence is observed in heart, lung, liver and pancreas transplant recipients. The lowest incidence (1-2%) is reported in renal transplant recipients (Allen *et al.*, 2013; Nijland *et al.*, 2016). At the ages of 5, 18, and 40, about 50, 20, 5-10% of the general population, respectively, have not been exposed to EBV, hence are seronegative. These groups of people develop primary infection after receiving transplant from a donor who is EBV seropositive, predisposing them to PTLD (Macsween *et al.*, 2003; Weikert *et al.*, 2008). The incidence of PTLD is more common in children than in adults as a result of increased pretransplant EBV seronegative status among children (Nijland *et al.*, 2016).

### 2.5.4 Nasopharyngeal Cancer (NPC)

Nasopharyngeal cancer is a malignant tumor of epithelial squamous cell which arises from the lateral wall of nasopharynx, notably, fossa of Rosenmüller and superior posterior wall (Sham *et al.*, 1990). The disease shows remarkable variation in ethnic and geographical distribution, with highest incidence being reported in Southern China and Asia. Globally, about 65,000 new cases of NPC are reported and 80% of these cases are reported from Southern China and Asia (Mimi *et al.*, 2002). The annual incidence rate of NPC in Southern China varies between 20-30 per 100 000 person-year among Cantonese living in Hong Kong and Guang-dong province, while the incidence rate in other part of the world such as Europe and the United States, is below 1 per 100 000 person-year (Chang *et al.*, 2006; Jia *et al.*, 2012). In Africa, specifically, Northern Africa, the incidence rate of NPC varies between 5 to 7 per 100 000 person-year (Hila *et al.*, 2009). In NPC endemic regions, the incidence peaks between ages 45 and 55, and declines thereafter. For reasons yet to be established, the rate of NPC cases is 2-3 folds higher among men than woman (Lo *et al.*, 2004). The variation in ethnic and geographical distribution of NPC indicates a multifactorial etiology, involving genetic susceptibility, EBV infection, ethnics, environmental factors and food consumption (Brennan, 2006; Li *et al.*, 2008). Based on histology, the World Health Organization (WHO), classified NPC into three types and these are types I, II, and III, which are known as keratinizing squamous cell, differentiated non-keratinizing, and undifferentiated non-keratinizing carcinomas, respectively. Type I is characterized by well differentiated keratin producing cells whereas types II and III are made up of non-keratin producing cells which are differentiated or undifferentiated, respectively (Shanmugaratnam, 1978). However, from etiological standpoint, an alternative, simpler system of classification has been proposed and this groups NPC into two histological variants, namely squamous cell carcinomas (SCCs) and undifferentiated carcinomas of the nasopharyngeal type (UCNT) (Micheau *et al.*, 1978). Undifferentiated carcinomas of the nasopharyngeal type is the prevalent histological type in endemic areas. For instance, in a non-endemic region like North America, 63% of all NPC cases are UCNT while in Southern China, 95% of all NPC cases are UCNT (Yoshizaki *et al.*, 2013). In a high risk region, UCNT is mainly EBV associated and based on that, EBV has been classified as group 1 carcinogenic agent by the International Agency for Research and Cancer (IARC) (Banko *et al.*, 2016). Epstein Barr virus infection in NPC epithelial cells is clonal in origin, strongly indicating that, NPC develops from clonal proliferation of single EBV infected epithelia cell. The association between EBV and NPC is based on the detection of elevated antibody titers against EBV related antigens such as EBV VCA, EBNA-1, and EA from the serum of NPC patients. In addition, NPC tumors have been found to express three EBV latency type II gene products such as LMP1, LMP2, EBNA-1, which are found in EBV implicated epithelial cancers, as well as the expression of viral encoded small RNAs such as EBER and microRNAs (miRNAs) (Banko *et al.*, 2016; Gao *et al.*, 2017; Wu *et al.*, 1991). As stated before, the infection of epithelia cells by EBV is mainly lytic and hence default lytic programs are expressed. However, the switch to latency default programs during epithelia infection by EBV, represents a key step in the pathogenesis of NPC. Although some studies have reported the involvement of both lytic and latent stages EBV genes in transformation of epithelia cells, the role of lytic genes still remains unclear (C.-P. Lee *et al.*, 2007; S.-D. Ma *et al.*, 2012; Sai-Wah Tsao *et al.*, 2015). Of all EBV latency gene products, LMP1 has been found in about two-thirds of NPC cases, indicating the key role this protein plays in tumorigenesis. Previous report involving clinical and follow-up data from 74 NPC cases, indicated that NPC cases positive for LMP1 grew faster and more expansively as compared to cases negative for LMP1 within a two-year period of follow-up (Hu *et al.*, 1995).

In addition to the above mentioned biological activities, LMP1 has been shown to drive host epigenetic changes to promote tumorigenesis. Reports have demonstrated that, in NPC cells, LMP1 downregulates the expression of E-cadherin, a tumor suppressor, through the activation of DNA methyltransferases (Tsai *et al.*, 2002). Despite the role of EBV in the NPC, the pathogenesis of the disease still remains enigmatic, as genetic, environmental and ethnic factors have also been linked to NPC (Tsao *et al.*, 2014).

### 2.5.5 EBV Associated Gastric Cancer (EBVaGC)

Gastric cancer (GC) is the third leading cause of cancer-related mortality globally, with a worldwide annual incidence of over 950 000 cases. EBV-associated gastric cancer (EBVaGC) accounts for about 10% of all GC reported cases (Jácome *et al.*, 2016; Shibata *et al.*, 1992). The incidence of EBVaGC shows variation in geographical distribution across the globe, with a pooled estimate in Europe, Asia, and North and South America, being 9.2%, 8.3% and 9.9%, respectively (Huang *et al.*, 2014), whereas in Africa, a country like Zambia has a frequency of 23% (Naseem *et al.*, 2018). EBVaGC is classified into three histological subtypes namely: lymphoepithelioma-like carcinoma (LELC)-type, conventional type adenocarcinoma (CA)-type, and carcinoma Crohn's disease- like lymphoid reaction (CLR)-type (Shinozaki-UshikuKunita *et al.*, 2015a)b. The LELC-type is a poorly differentiated carcinoma with dense infiltration of lymphocytes, similar to that of NPC. More than 80% of EBVaGCs show LELC-type morphology (van Beek *et al.*, 2004). CA-type on the other hand, morphologically resembles EBV negative GCs by infiltration of variable lymphocytes with prominent desmoplasia in the absence of lymphoid follicles (Song *et al.*, 2011). CLR-type is characterized by the presence of three or more lymphoid follicle with active germinal centers located at the advancing edge of the neoplasm (Song *et al.*, 2011)

One of the unique features which distinguishes EBVaGC from non-EBVaGC is the monoclonal proliferation of gastric epithelia cells latently infected with EBV, and suggesting the presence of the virus in the early stages of tumorigenesis (Uozaki *et al.*, 2008). In contrast to EBV-negative GCs, which mainly occur in the antrum as the predominant pathological site, EBVaGC occurs predominantly in the proximal stomach including the cardia, fundus and body (Song *et al.*, 2011). At early stages of tumorigenesis, EBVaGC forms a well-defined nodular ulcer in the submucosa with less fibrosis as compared to non-EBVaGCs, and this pathological feature is essential for endoscopic submucosal resection of the tumor (Lee *et al.*, 2014).

The genome of EBV, mainly Latency I default programs (EBER, EBNA-1, and BART), was found in the GC cells and adjacent dyspalstic epithelium cells, but could not be found in surrounding lymphocytes, stromal cells, normal mucosa, and intestinal metaplasia, and therefore suggests a key role of EBV in the pathogenesis of GC (Shinozaki-UshikuKunita & Fukayama, 2015a).

### 2.5.6 Extranodal Nasal-type T/NK Lymphoma

Nasal-type T/NK lymphomas are infrequent tumors that are regularly associated to EBV infection (Neparidze *et al.*, 2014). The lymphoma occurs as a result of malignant transformation of NK cells with CD 2, CD 56 and TIA-1, as well the absence of CD3 gene rearrangement of the T cell lymphocyte receptor (T. Zhang *et al.*, 2014). The tumor is characterized by extensive angio-invasion and necrosis occurring in upper aerodigestive tract including nasal cavity, nasopahrynx, paranasal sinus and palate (Chan, 2008). About 20% of the cases presented at extra-nasal sites such as skin, testis, respiratory and gastrointestinal tracts. Presentation on the skin is marked by nodular lesion which is usually ulcerative, and gastrointestinal bleeding/ intestinal perforation are characteristic of gastrointestinal tract presentation (Wong *et al.*, 1992). The disease shows remarkable variation in geographical and sex distribution. Highest incidence occurs in East Asia, Mexico, South and East America. It has also been reported that, this type of lymphoma is very predominant among men than women, with 2-3:1 ratio of male to female, and the mean age at diagnosis being 50 years (Au *et al.*, 2009; Chim *et al.*, 2005).

Epstein Barr virus expresses type II latency programs (EBNA-1, LMP-1 and -2) in the T/NK lymphoma cells, and the genome exists as a clonal episome in these cells (Dojcinov *et al.*, 2018). The expression of these genes has been shown to constitutively activate several signaling pathways including JAK/STAT and NF-κB, which promote cell growth and survival (Jiang *et al.*, 2015). More recent study has also reported that LMP1 expression in T/NK lymphoma cells promote tumor immune escape through the up-regulation of programmed cell death receptor 1 (PD-1) and PD ligand 1, which are important immune checkpoint molecules in tumor immunology (Bi *et al.*, 2016).

Genome wide analysis have reported chromosomal abnormalities, notably, deletions in 6q21 in T/NK lymphoma cells (Nakashima *et al.*, 2005). Deletion in 6q21 leads to the loss of expression of many tumor suppressor genes such as ATG5, PRDM1, FOXO3, AIM1, and HACE 1. For instance, the loss of FOXO3 and HACE1 in EBV+ T/NK lymphoma cells results in apoptosis resistance by respectively, preventing the induction of BIM and PUMA, and by suppressing TNF-driven NF-κB activation (Huang *et al.*, 2013).

### 2.5.7 Breast Cancer

Breast cancer is the most frequently diagnosed female malignancy globally and accounts for about 25% of incident cancer cases among women (Shadmani *et al.*, 2017). In underdeveloped regions such as sub-Saharan Africa, frequency of breast cancer is relatively lower, characterized by aggressive course and targets females of younger age compared to the Western World (Fregene *et al.*, 2005).

While genetic factors such as the inheritance of breast cancer associated gene 1 and 2 (*BRCA1/2*) and human epidermal growth factor receptor 2 (*HER2*) have been reported to influence the development of breast cancer, with other factors such as sex, lifestyle, and reproductive system, reported to have increased the development of this malignancy, the etiology still remains unknown (Sun *et al.*, 2017). Other agents including EBV, mouse mammary virus (MMV), human papilloma virus (HPV), and cytomegalovirus (Glenn *et al.*, 2012; Pasquereau *et al.*, 2017; Salman *et al.*, 2017) have been implicated in breast cancer cases. Association between EBV and breast cancer was reported from different parts of the world including Asia (Joshi *et al.*, 2009), Africa (Hachana *et al.*, 2011), and Europe (Marrão *et al.*, 2014). Other studies have also reported that about 30-50% of breast cancer cases globally are EBV associated (Glenn *et al.*, 2012; Lorenzetti *et al.*, 2010). Following these reports, many researchers have also detected EBV latency II gene products such as LMP-1, -2, and EBNA-, as well as EBER from breast cancer cells, strengthening the link between EBV and this malignancy (Arbach *et al.*, 2006; Fessahaye *et al.*, 2017; Lorenzetti *et al.*, 2010). Previous study has reported that transfection of p31 DNA of EBV immortalized epithelia cells including mammary epithelia cells and this observation suggested that breast cancer is a malignancy of epithelia origin (Glaser *et al.*, 2004)

Despite the detection of EBV antigens in breast cancer cells, there is conflicting evidence regarding the role of the virus in the pathogenesis of the cancer. Cox and colleagues have reported that EBV infection is not associated to the risk of developing breast cancer (Cox *et al.*, 2010). On the other hand, Yasui and colleagues have previously reported that delayed EBV infection, that is, infection occurring during adolescent or adulthood stages, was associated to breast cancer (Yasui *et al.*, 2001). Recently, a meta-epidemiological studies have reported that EBV infection was highly associated to the risk of breast cancer development (Bae *et al.*, 2016). The inconsistencies of these reported findings have been attributed to variation in geographical distribution of viral and host genomes, usage of different techniques, socioeconomic and racial variations ( Lorenzetti *et al*., 2012).

# CHAPTER THREE

## 3.0 Materials and Methods

## 3.1 Chemicals and Reagents

Qiagen DNeasy blood and tissue kit and QIAamp Blood Mini Kit used for extraction of DNA from biopsies and blood samples, respectively, were obtained from QIAGEN, Hilden, Germany. One *Taq*$^{R}$ Quick-Load 12X Master Mix with Standard Buffer was purchased from New England BioLabs, UK. Primers for amplification of EBNA-1, EBNA-2, LMP-1 and Beta-globulin were purchased from Integrated DNA Technologies (IDT), USA. Agarose powder, Luria Broth agar, 50X Tris-acetate -EDTA (TAE) Buffer, Ampicillin, Ethanol, Glucose, Tris-HCl, NaOH, SDS, and Potassium Acetate were obtained from Sigma-Aldrich, USA. PerFecta SYBR Green SuperMix Low Rox was obtained from Quantabio (USA). Top10 competent *Escherichia coli* (*E. coli*) cells, pJET-EBNA-1 pJET-EBNA-2 (EBV types 1 and 2) and pJET-Beta globulin plasmid were obtained from University of Sussex, UK.

### 3.1.1 Equipment

Amersham Gel Imager was obtained from GE Healthcare, United States. Nanodrop Spectrophotometer$^{TM}$ , Precision$^{TM}$ Circulating Water Bath, Heraeus™ Pico™ 17 Microcentrifuge were obtained from Thermo Fisher Scientific, United States. QauntStudio5 PCR System was obtained from Applied Biosystem, United States.

## 3.2 Study Site

The study site was the Ear, Nose, and Throat (ENT) Unit of the Korle-Bu Teaching Hospital. This Unit serves as a referral center for all nasopharyngeal cancer (NPC) cases in Ghana and West Africa.

## 3.3 Study Design and Target Population

The study was a laboratory-based cross-sectional study on NPC tumor biopsies and blood samples from NPC patients yet to undergo treatment at the ENT Unit of KBTH, as target population. Blood samples were collected from NPC negative individuals and used as controls. Well-structured questionnaire was administered to acquire demographic data and risk factors from the study participants after informed consent has been obtained

### 3.3.1 Sample Size Calculation

The prevalence of NPC as reported by Kitcher *et al* (2004) was 1.2-1.3%. Base on this, the sample size will be determined using the formula below: $n = p(1-p)/e^2$, where p is the prevalence, n is the sample size and e is the error margin. Therefore $n= 0.012(1-0.012)/0.01^2 = 118$.

## 3.4 Inclusion and Exclusion Criteria

Subjects enrolled in this study were patients diagnosed of NPC by endoscopy and computed tomography (CT) scan and were yet to undergo treatment. Subjects who were confirmed NPC negative or without any known oncological disease by endoscopy and CT scan were also enrolled and used as negative control. Patients who were on treatment or failed to give their consents were excluded from the study.

## 3.5 Ethical Approval

The study protocol was approved by the Institutional Review Boards of Noguchi Memorial Institute for Medical Research (Protocol number: 051/16-17), Korle-Bu Teaching Hospital (Protocol number: KBTH-STC 00020/2018). Clinical samples used in this study were agreed on by the ENT Unit of the Korle-Bu Teaching Hospital after each participant signed consent form.

### 3.6 Plasmids and Transformation of *E. coli* Cells

One Shot Top10 competent *Escherichia coli* (*E. coli*) cells were transformed with the following plasmids: pJET-EBNA-1, pJET-EBNA-2 (EBV types 1 and 2) or pJET-Beta globulin and used as positive controls. An aliquot of 30 µL of competent *E. coli* cells were pipetted into prechilled 1.5 ml micro centrifuge tubes and 1 µL of 10 ng/µl pJET-EBNA-1, pJET-EBNA-2 (EBV types 1 and 2), pJET-Beta globulin or pJET-LMP-1 plasmids were added, incubated on ice for 30 minutes. The tubes were removed from the ice and incubated in water bath for 40 seconds at 42 $^0$C after which 100 µL of SOC media was added and immediately transferred onto ice for 2 minutes. The tubes containing the mixture were again incubated in a water bath at 37 $^0$C for 1 hour, whiles shaking was done at 180 rpm. A volume of 50 µL of the transformation mix was spread on solidified agar supplemented with 20 µL of ampicillin and overnight incubation was done at 37 $^0$C. A control having *E.coli* cells spread on agar plates supplemented with or without ampicillin was set up. Singles colony of transformed cells growing on the test plate was picked and grown overnight in 3 ml of Luria Broth containing 3 µL of ampicillin.

### 3.7 Extraction of Plasmid DNA from Transformed Cells

To extract plasmids from transformed cells, 1.5 ml of overnight culture of transformed *E.coli* cells was centrifuged at 10,000 rpm after which the supernatant was discarded. The pellet was resuspended in 200 µL of Solution I (Glucose, Tris-HCl, and EDTA) and mixed thoroughly by vortexing, followed by the addition of 200 µL of Solution II (NaOH and SDS) and homogenization was done by inverting the mixture gently. A volume of 200 µL of Solution III (Potassium Acetate) was then added, inverted and centrifugation was carried out at 10,000 rpm for 10 minutes. The supernatant was transferred into a 1.5 ml fresh tube and 900 µL of ice cold 100% ethanol was added and the tube inverted several times, followed by centrifugation at 10,000 rpm for 20 minutes at 4 $^0$C. The supernatant was discarded and 100 µL of ice cold 75% ethanol was added to the pellet and further centrifuged at 10,000 rpm for 30 seconds at 4 $^0$C. The supernatant was discarded and the pellet was air-dried for 30 minutes to remove all residual ethanol. The air-dried DNA pellet was resuspended in 100 µL of nuclease free water and quantified using Nanodrop Spectrophotometer$^{TM}$.

## 3.8 Sample Collection

### 3.8.1 Collection of Blood Samples

A volume of 5 ml venous blood from 55 NPC patients and 53 NPC negative individuals were collected using 5 ml syringe into EDTA treated tubes and transported to the Virology Laboratory, Department of Biochemistry, Cell and Molecular Biology, University of Ghana, and temporarily stored at 4 $^0$C for subsequent analysis.

### 3.8.2 Collection of NPC Biopsies

Both nasal cavities of NPC patients were sprayed or packed with cotton wool soaked in a topical and vasoconstrictive solution. The nasopharynx was systematically examined with a rigid endoscope (0 or 30 degree), and if a definite mass/lesion was seen in the nasopharynx, a biopsy of the nasopharyngeal mass/lesion was taken under local anaesthesia (LA).The procedure was aided by healthcare workers at the ENT Unit of the Korle-Bu Teaching Hospital.

The biopsies were place in 2 ml Eppendorf tubes filled with formalin and transported to the Virology Laboratory, Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

### 3.8.3 Extraction of DNA from Blood Samples

Genomic DNA was extracted from blood samples from NPC patients and NPC negative individuals using QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany) by following the manufacturer's protocol. An aliquot of 200 µL of whole blood sample was added 2 ml microcentrifuge tube which contained 20 µL of proteinase K and this was followed by the addition of 200 µL of Buffer AL and vortexing. The mixture was pulse-spun and incubated at 70 ºC for 20 minutes. A volume of 200 µL of ethanol (96-100%) was added to the sample, mixed by vortexing and applied to QIAamp Mini spin Column without wetting the rim. The column was centrifuged at 8000 rpm for 1 minute and placed in a clean 2 ml collection tube after discarding the filtrate. An aliquot of 500 µL of Buffer AW1 was added and the column was centrifuged at 8000 rpm for 1 minute. After spinning, the column was transferred into another clean 2 ml collection tube and the filtrate discarded. Buffer AW2 in a volume of 500 µL was added and the column centrifuged at full speed for 3 minutes. The column was transferred into a clean 1.5 ml microcentrifuge tube and the filtrate in the collection tube was discarded. Centrifugation at full speed for 1 minute was done and the column was placed in another clean 1.5 ml microcentrifuge tube. DNA was eluted with 200 µL of Buffer AE after incubation at room temperature for 30 minutes. The eluted DNA samples were quantified using Nanodrop Spectrophotometer$^{TM}$ and stored at -20$^{0}$C for further analysis.

### 3.8.4 Extraction of DNA from NPC Biopsies

DNA from biopsy tissues was extracted using Qiagen DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) by following the manufacturer's instructions with slight modification. Biopsy tissues were cut into small pieces and ground in 1 ml of PBS using pestle and mortar. The paste was transferred into 2 ml microcentrifuge tubes and centrifugation was carried out at full speed.

The supernatant was discarded and 180 µL of buffer ATL and 20 µL of proteinase k were added and mixed thoroughly by vortexing. The mixture was incubated overnight at 56 $^{0}$C in thermal block. After incubation, the mixture was vortexed for 15 seconds and 200 µL of buffer AL was added, followed by vortexing. Aliquot of 200 µL of ethanol (96-100%) was added and mixed thoroughly by vortexing. The mixture was pipetted into DNeasy mini spin column placed in 2 ml collection tube and centrifuged at 6000 x g for 1 minute. The flow-through and the collection tube were discarded and the DNeasy spin column was placed in a new 2 ml collection tube and 500 µL of buffer AW1 was added, and centrifuged for 1 minute at 6000 x g. After discarding the flow-through and the collection tube, the spin column was placed in a new 2 ml collection tube and 500 µL buffer AW2 was added and subjected to centrifugation at 20,000 x g for 3 minutes to dry the membrane. DNA was eluted with 200 µL of buffer AE, quantified and stored at -20 $^{0}$C for further analysis.

### 3.8.5 Epstein Barr Virus (EBV) Detection by EBNA-1 Amplification

The presence of EBV was screened in DNA extracted from blood and biopsy samples by Polymerase chain reaction (PCR) amplification of EBNA-1, with primers reported by Lay *et al.* (2010) with slight modification to cycling conditions. The sequences QP1: GCC GGT GTG TTC GTA TAT GG and QP2: CAA AAC CTC AGC AAA TATATG AG were used as forward and reverse primers for EBNA-1 gene. Beta globulin gene was also amplified and used as internal control for gene amplification and to confirm the absence of PCR inhibitors, using sequences GH 21R: GGAAAATAGACCAATAGGCAG and GH21F: GAAGAGCCAAGGACAGGTAC, as forward and reverse primers, respectively. Positive controls for EBNA-1 and beta globulin genes were plasmids containing EBNA-1 and beta globulin, respectively. A PCR reaction of total volume 12.5 µL, consisting the following components, 50 ng of template DNA, One *Taq*$^{R}$ Quick-Load 12X

Master Mix with Standard Buffer, 10 µM each of QP1,QP2, GH 21R and GH21F, and nuclease free water was set up. The cycling conditions for the PCR reaction were as follow: Initial denaturation at 94 ºC for 30 seconds, followed by 35 cycles of amplification with denaturation for 30 seconds at 94 ºC, annealing for 1 minute at 56 ºC, extension for 1 minute at 68 ºC and a final extension for 5 minutes at 68 ºC. An aliquot of 8 µL of the amplicons were resolved on 2% ethidium bromide- stained agarose gel and bands were visualized using Amersham gel imager. The expected band sizes were 213 bp and 110 bp for EBNA-1 and beta globulin, respectively.

### 3.8.6 Identification of EBV Types by EBNA2 Typing

The genotypes of EBV present in blood and biopsy specimen were detected by nested PCR using protocol previously described by Hassan *et al.* (2006) with slight modifications to cycling conditions. The first round of the PCR was done by amplifying a common region of EBNA-2 using the primers EBNA-2F (TGGAAACCCGTCACTCTC) and EBNA-2I (TAATGGCATAGGTGGAATG) as sense and antisense primers, respectively. Amplification was done in 12.3 µL reaction volume containing 50 ng of template DNA, One *Taq*$^R$ Quick-Load 12X Master Mix with Standard Buffer, 10 µM each of EBNA-2F and EBNA-2I, and nuclease free water. The cycling conditions for the reaction were as follow: Initial denaturation at 94 ºC for 2 minutes, followed by 35 cycles of amplification with denaturation for 60 seconds at 94 ºC, annealing for 90 seconds at 52 ºC, extension for 4 minutes at 72 ºC and a final extension for 10 minutes at 72 ºC. A second round PCR (nested) was performed using 0.5 µL of the amplicons from the first round PCR as template with all other constituents remaining the same with the exception of the primers. A forward primer (EBNA-2C: AGGGATGCCTGGACACAAGA) common to both genotypes and reverse primers (EBNA-2G: GCCTCGGTTGTGACAGAG, EBNA-2B: TTGAAGAGTATGTCCTAAGG) specific for genotypes 1 and 2, respectively, were used. The reaction was carried out at initial denaturation of 94ºC for 2 minutes, followed by 35 cycles of amplification with denaturation for 30 seconds at 94 ºC, annealing for 60 seconds at 52 ºC, extension for 2 minutes at 72 ºC and a final extension for 10 minutes at 72 ºC. Ten microliters (10 μL) of the amplicons were resolved on 2% ethidium bromide-stained agarose gel and visualization was done using Amersham gel imager. The expected band sizes are 250 bp and 300 bp for genotypes 1 and 2, respectively.

### 3.8.7 Determination of EBV DNA Load by Real-Time PCR

Epstein Barr virus DNA load in the tumor biopsies and blood samples were determined by relative quantification method using real-time PCR (QauntStudio5 PCR System, Applied Biosystem), by amplifying Epstein nuclear antigen 1(EBNA-1), a conserved gene which is expressed in all NPC cells. PCR reaction volume of 10 μL containing the following components: 5 μL 1X Sybr Green SuperMix, template DNA, 0.1 μL of 0.1 μM each of target primer (sense, QP1: GCC GGT GTG TTC GTA TAT GG and antisense, QP2: CAA AAC CTC AGC AAA TATATG AG), and 3.8 μL of nuclease free water. Each reaction was normalized by amplification of betaglobin gene, to correct inter sample or plate-to-plate variation. The cycling conditions consisted of a 10-min hold at 95 °C, followed by a first denaturation step of 10 minutes at 95 °C, and then 40 cycles of 95 °C for 15 seconds (denaturation) and 60 °C for 1 minute (reannealing and extension). All samples were analyzed in replicates and after amplification, melting curve analyses was conducted to confirm that only specific products were amplified.

### 3.8.8 Construction of Standard Curve for EBV DNA Load Quantification

To extrapolate the unknown concentration of EBV load in the various blood and biopsy samples, a standard curve was constructed using a known concentration of EBV DNA. pJET plasmid containing EBNA-1 gene was spectrophotometrically quantified and EBV copies were calculated following the formula: $copy\ number = \frac{\text{Amount of plasmid(ng)}\times 6.0221\times 10e23}{\text{plasmid length (bp)}\times 650\ (\frac{g}{mol})\times 10e9}$. A 10-fold serial dilution of the stock pJET-EBNA-1 of concentration $10^{11}$ copies/ µL was prepared after which working dilutions of $10^{8}$ to $10^{10}$ were made. The serially diluted pJET-EBNA-1 plasmids were run in replicate to generate cycling threshold ($C_T$) values for each dilution. Standard curve was made by plotting the $C_T$ values against the log concentration of each dilution.

### 3.9 Statistical Analysis

Statistical analysis was done using IBM SPSS statistical software version 20 and GraphPad Prism 8. Differences in the distribution of demographic parameters and risk factors between NPC cases and control subjects were evaluated using Chi-squared test of independence for contingency table and corrected by Yates' continuity test or Fisher Exact test, when needed. Degree of association was estimated using odds ratio (OR) with 95% confidence interval. After confirming the normal distribution of EBV load results by Kolmogorov and Smirnov tests, Mann Whitney rank-sum test was used to compare median viral load between cases and controls for binary categories or Kruskal- Wallis test for more than two categories. A p-value less than 0.05 was considered statistically significant.

# CHAPTER FOUR

## 4.0 Results

### 4.1 Demographic Parameters of Study Subjects

A total of 108 study participants reporting to the ENT Units of KBTH from April 2018 to March 2019 were enrolled in the current study. Of the 108 study participants, a total of 55 of the participants were diagnosed of NPC by endoscopy and computed tomography (CT) scan and 53 were confirmed NPC negative or without known oncological diseases by CT scan and endoscopy, hence served as negative controls. The median age of the cases (NPC patients) was 40 (range 12-79) and that of the control subjects was 47 (range10-80). The frequencies of males and females diagnosed with NPC were 47% (26/55) and 53% (29/55) respectively, giving male to female ratio of 1:1. In control samples, the frequencies were 30% (16/53) and 70% (37/53) for males and females, respectively (Table 4.1).

### 4.2 Frequency of NPC across Age Categories

The frequency of NPC was determined across three age categories, 10 to 30, 31 to 61, and 62 to 92 years. The highest frequency (44 %) of NPC was seen in patients within the ages of 31 to 61 years and the least frequency (18%) was observed in patients between the ages of 62 to 92 years (Figure 4.2)

### 4.3 Regional Distribution of NPC Cases in Ghana and Neighboring Countries

As shown in Figure 4.2, The Ear, Nose and Throat Unit of the Korle-Bu Teaching Hospital received NPC cases from 8 regions in Ghana and 2 neighboring countries, namely, Liberia and Togo, between May 2018 and March 2019. Out of the 8 regions in Ghana, majority (31%) of NPC cases were reported from the Eastern region of Ghana and the lowest frequency (1.8%) of cases was reported from Northern, Upper East and Ashanti regions. The frequency of NPC cases received from Togo and Liberia was 1.8%.

### 4.4 Evaluation of Risk Factors Associated to NPC Development in Subjects

In this study we evaluated the association of gender, alcohol intake, smoking, and regular consumption of salt-preserved fish with NPC. It was observed that gender, alcohol intake, and smoking were not associated with NPC development, however regular consumption of salt-preserved fish was strongly associated with NPC development (Tables 4.2 and 4.3).

### 4.5 EBV Detection by EBNA-1 Amplification in Blood and Biopsy Specimen

As depicted in Figure 4.3, EBV was detected in the various clinical specimen from cases and control by amplification of EBNA-1. EBNA-1 forward and reverse primers yielded a PCR amplicon of size 213 bp when resolved on 2% agarose gel. EBV positivity was estimated by comparing positivity in samples from cases with control blood (Figure 4.4).

### 4.6 Evaluation of Risk of EBV Infection by Gender

In this study the association of EBV infection with gender was investigated by comparing EBV positivity in blood samples from male participants to that of female participants. There was no significant association of gender with the risk of EBV infection ($\chi2 = 1.770$, $p = 0.1834$), as depicted in Table 4.4.

### 4.7 Characterization of EBV Genotypes by Amplification of EBNA-2 in Blood and Biopsy Specimen

From Figure 4.5, EBV genotype identification was carried out by EBNA-2 amplification in specimen from cases and control, using genotype specific primers. EBV genotypes 1 and 2 amplification using genotype specific primers yielded band sizes of 250 bp and 300 bp characterized genotypes 1 and 2, respectively.

As shown in Figure 4.6 A, frequency of EBV genotype 1 was 12% (6/52) in blood samples from NPC cases, and 62% (33/53) in blood samples from control subjects, making genotype 1 the predominant genotype found in blood of healthy carriers. The frequency of genotype 2 was highest (52% (27/52)) in blood samples from NPC cases as compared to the frequency found in control blood samples which was 6% (3/53). Hence the predominant genotype in the blood of NPC patients was EBV genotype 2. The frequencies of co-infection with genotypes 1 and 2 was 30% in both NPC blood (16/52) and control blood (16/53) samples. Statistically significant difference ($\chi2$= 72.26, p = 0.001) was observed for the frequency of EBV genotypes in the NPC blood and controls. Frequencies of 6% (3/52) and 2% (1/53) of case blood and control blood samples, respectively, tested negative for EBV typing. For NPC biopsies the frequency of EBV genotype 1 was 58% (32/55), that of genotype 2 was 4% (2/55), and co-infection with genotypes 1 and 2 was 38% (21/55). Hence in NPC biopsies the predominant genotype was EBV genotype 2 (Figure 4.6 B).

### 4.8 Determination of Frequency of EBV Genotypes among Male and Female Participants

In this study EBV genotype profile in blood samples of male and female participants was investigated. In the blood of male participants, genotypes 1 and 2, were equally distributed with each having frequency of 31% (13/42). The frequency of co-infection among the male participants was also 31% (13/42), and no genotype was observed in blood sample of 3 male subject (7%). In the case of female subjects, frequencies of 39.4% (26/66), 28.8% (19/66), and 25.8% (17/66) were observed for EBV genotypes 1 and 2, and genotype 1 and 2 coinfection, respectively. In this study, the predominant EBV genotype in female participant was EBV genotype 1. Typing result was negative in 4 out of 66 (6%) blood samples from female participants (Figure 4.7)

### 4.9 Distribution of EBV Viral Load in NPC Case and Control Specimen

Epstein Barr DNA load in NPC biopsies and whole blood samples from NPC patients and control participants was determined by EBNA-1 real-time PCR. The viral load results were not normally distributed, hence Mann-Whitney or Kruskal-Wallis test were used to analyze median viral load distribution between NPC cases and control subjects after expressing the results as $\log_{10}$EBVDNA copies/mL. A p-value less than 0.05 was considered statistically significant. EBV load results of samples from NPC cases and control subjects are shown in Figures 4.8, 4.9, 4.10 and 4.11

The range of EBV DNA load (copies/ml) in NPC cases was from $53\times10^3$ to $5\times10^{11}$ copies/ mL with median viral load value of $9\times10^7$ copies/ mL. In control subjects, the median viral load value was $6\times10^4$ copies/ mL, ranging from $4\times10^2$ to $8\times10^6$ copies/ mL. The median EBV DNA load was significantly higher in NPC cases compared to control subjects ($p=0.001$), when estimation was done using Mann-Whitney test (Figure 4.8).

In the current study, median EBV load in NPC blood samples was compared to that of NPC tumor biopsies using Mann-Whitney test. The median EBV load value in NPC blood was $9\times10^7$ copies/ mL which ranged from $53\times10^3$ to $5\times10^{11}$ copies/ mL. NPC tumor biopsies had median EBV DNA load of $1.58\times10^5$ copies/mL ranging from 618 to $1.2\times10^7$ copies/ mL. Significantly higher ($p < 0.001$) median EBV DNA load was observed in NPC whole blood samples compared to NPC tumor biopsies (Figure 4.9)

The distribution of median EBV DNA load was further evaluated in NPC whole blood samples, NPC biopsies and whole blood samples from control subjects harboring different genotypes of EBV. As shown in Figure 4.10, there was no significant difference ($p > 0.05$) in the distribution of

EBV DNA load in whole blood samples from NPC patients and control subjects as well as tumor biopsies, infected with one or two genotypes of EBV, when comparison was done using Kruskal-Wallis test.

Median EBV DNA load in NPC cases with mono or co-infection of EBV genotypes was compared to mono or coinfected control samples using Mann-Whitney test. From Figure 4.11, the median viral load of case samples infected with EBV genotype 1 was significantly higher ($p = 0.001$) than control samples infected with the same EBV genotype (genotype 1 median viral load in cases, 2 $\times 10^7$ copies/ mL verses 29 $\times 10^3$ copies/ mL genotype 1 median viral load in controls) (Figure 4.11 A). Significantly higher ($p = 0.001$) median viral load was observed in cases infected with EBV genotype 2 when compared to control samples infected with the same EBV genotype ($1\times 10^8$ copies/ mL compared to 18 $\times 10^3$ copies/ mL in cases and controls, respectively) (Figure 4.11 B). The same trend was observed when median viral load in NPC cases coinfected with EBV genotypes 1 and 2 was compared to that of controls with co-infection ($1\times 10^8$ copies/ mL compared to79 $\times 10^3$ copies/ mL in cases and controls, respectively) (Figure 4.11 C).

**Table 4. 1: Demographic parameters of nasopharyngeal cancer (NPC) patients and control subjects**

| **Variable** | **Patients diagnosed with NPC** (N=55) | **Controls** (N=53) |
|---|---|---|
| Age (yrs) | 40 (range 12-79) | 47 (range10-80) |
| Sex: n (%) | | |
| Male | 26 (47) | 16 (30) |
| Female | 29 (53) | 37 (70) |

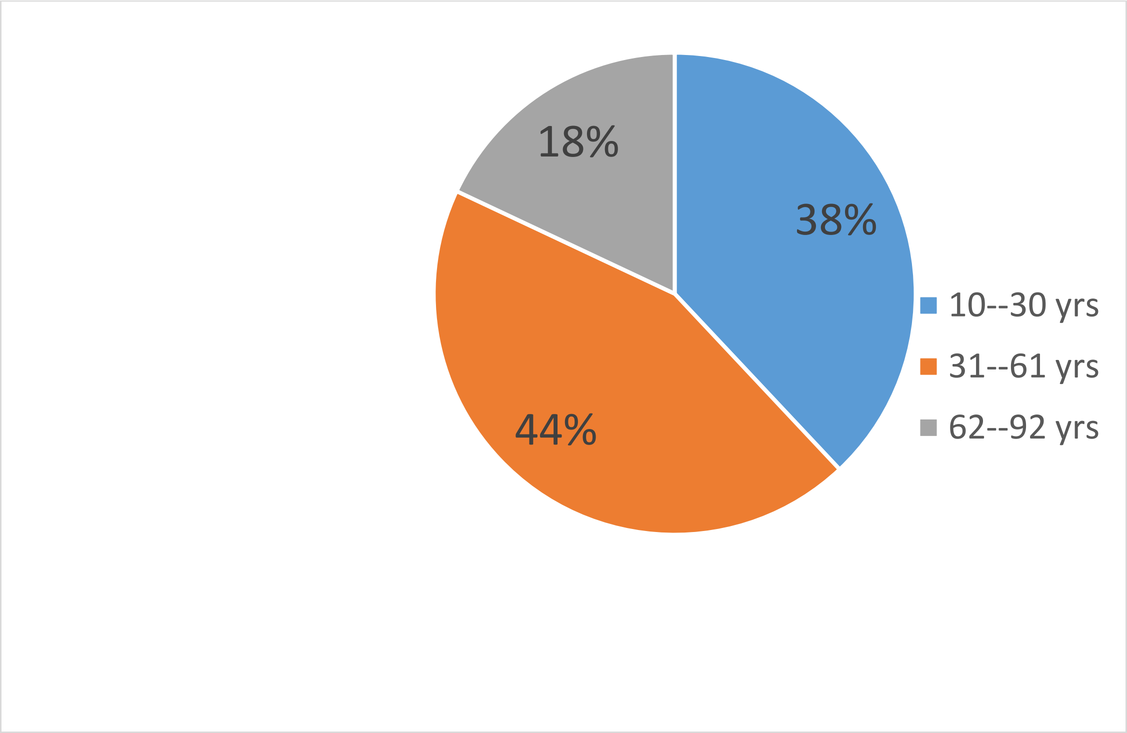

**Figure 4. 1. Distribution of NPC frequency across different age categories**

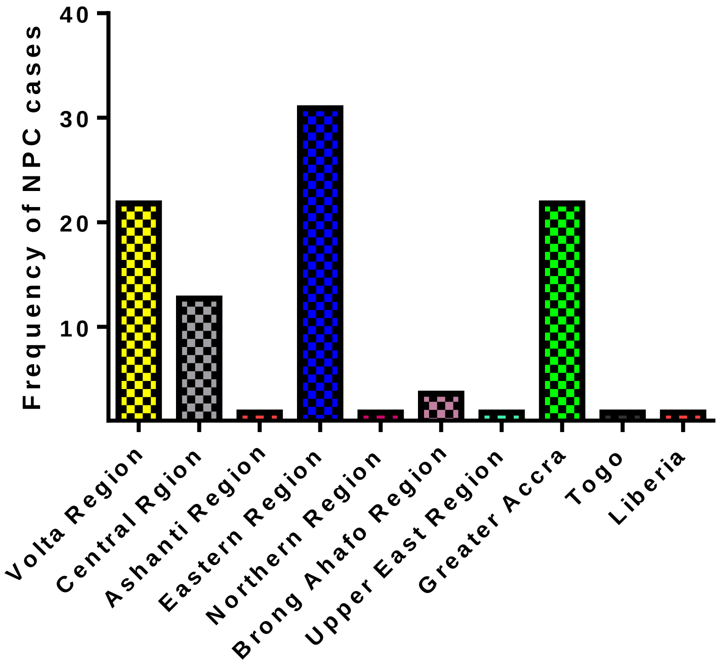

**Figure 4. 2. Geographical distribution of NPC cases across 8 regions in Ghana and two neighboring countries in Africa**

**Table 4. 2: Risks for NPC development by gender and lifestyle**

| | N (%) | | | |
|---|---|---|---|---|
| **Risk factor** | **Nasopharyngeal cancer** | **Control subject** | **χ2** | **p-value** |
| **Gender** | | | | |
| Male | 26 (62) | 16 (38) | 2.635 | 0.1045 |
| Female | 29 (44) | 37 (56) | | |
| **Alcohol consumption** | | | | |
| Take alcohol | 13(50) | 13(50) | 0.01362 | 0.9071 |
| Do not take alcohol | 42 (51) | 40 (49) | | |
| **Smoking status** | | | | |
| Smoked | 3 (43) | 4(57) | 0.0026 | 0.9596 |
| Do not smoke | 52 (51) | 49 (49) | | |
| **Salted fish consumption** | | | | |
| Take salted fish | 48 (58) | 35(42) | 5.7000 | 0.0170* |
| Do not eat salted fish | 7 (28) | 18(72) | | |

***Difference was statistically significant when estimated using Chi-squared test corrected by Yates' continuity test**

**Table 4. 3: Odds of NPC development by salted fish consumption**

| | N (%) | | | | |
|---|---|---|---|---|---|
| **Risk factor** | **Nasopharyngeal cancer** | **Control subject** | **OR** | **95%CI** | **p-value** |
| **Salted fish consumption** | | | | | |
| Take salted fish | 48 (87) | 35(66) | 3.527 | 1.329- 9.358 | 0.0170* |
| Do not eat salted fish | 7 (13) | 18(34) | | | |

**Abbreviations:** OR, odds ratio; CI, confidence interval.

***Difference was statistically significant.**

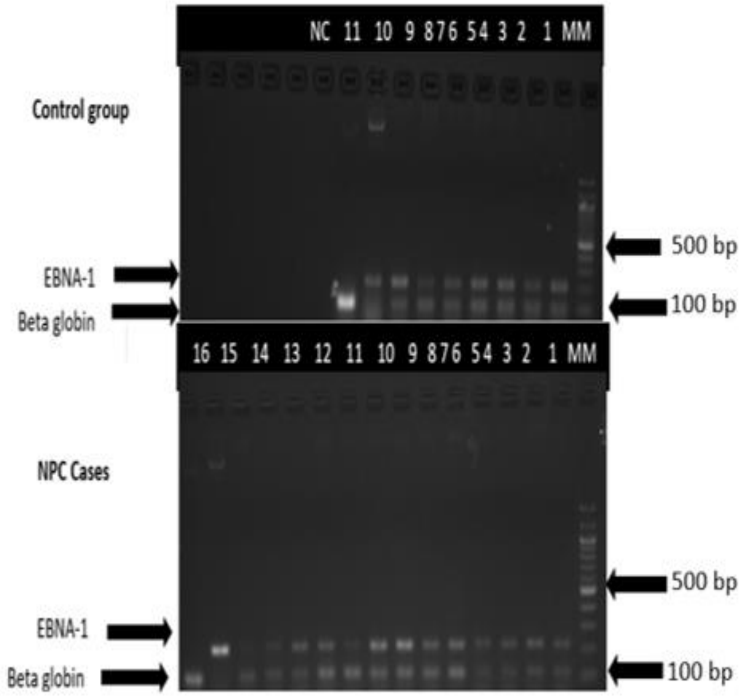

**Figure 4. 3. Detection of EBV in cases and control specimen by amplification of EBNA-1 gene. NPC cases:** lanes 1 to 14 were amplified EBNA-1 gene, lane 15 and 14 were EBNA-1 and Beta globin positive controls, respectively. **Control groups**: lanes 1 to 9 were amplified EBNA-1 gene. Lanes 10 and 11 were EBNA-1 and Beta globin positive controls, respectively. NC: Negative control. Expected band sizes of EBNA-1 and Beta globin were 213 bp and 110 bp, respectively. MM: 100 bp molecular weight marker.

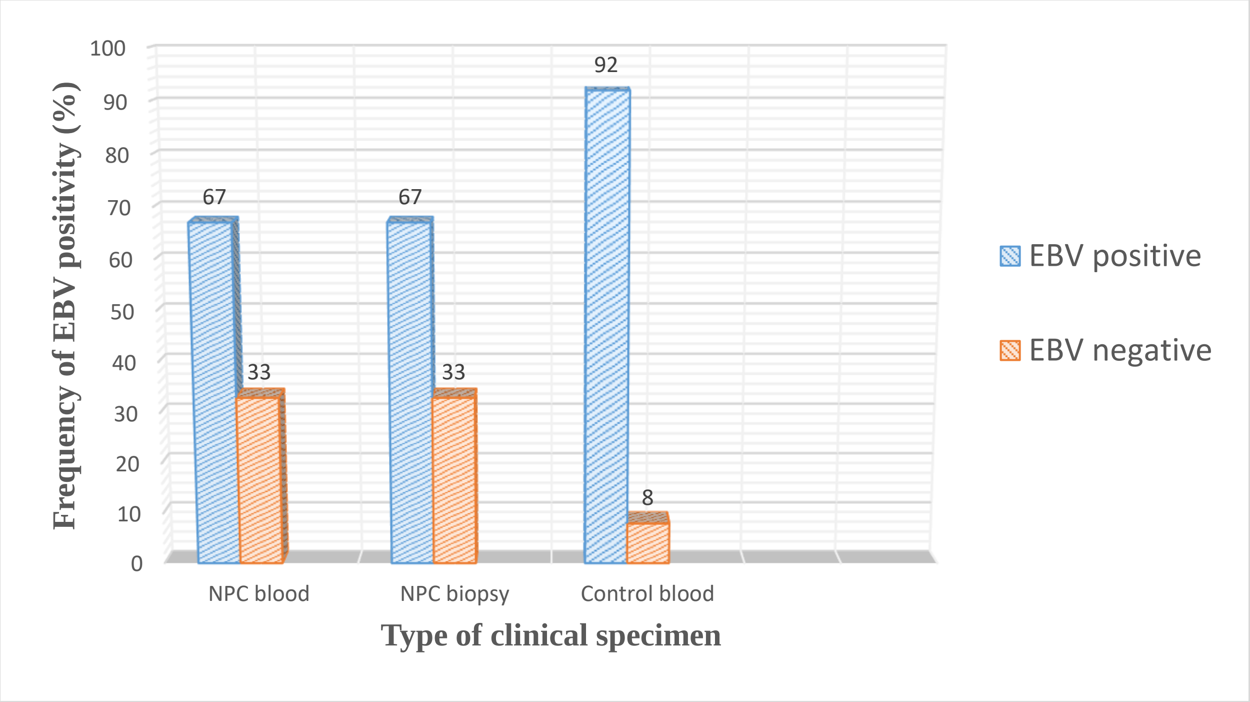

**Figure 4. 4. Frequency distribution of EBV positivity in NPC cases and control**

**Table 4. 4: Association between gender and risk of EBV infection**

| | **EBV Positivity** | | | |
|---|---|---|---|---|
| **Risk factor** | **EBV Positive** | **EBV Negative** | **χ2** | **p-value** |
| **Gender** | | | | |
| Male | 28 (70) | 12 (30) | 1.770 | 0.1834 |
| Female | 54 (83) | 11 (17) | | |

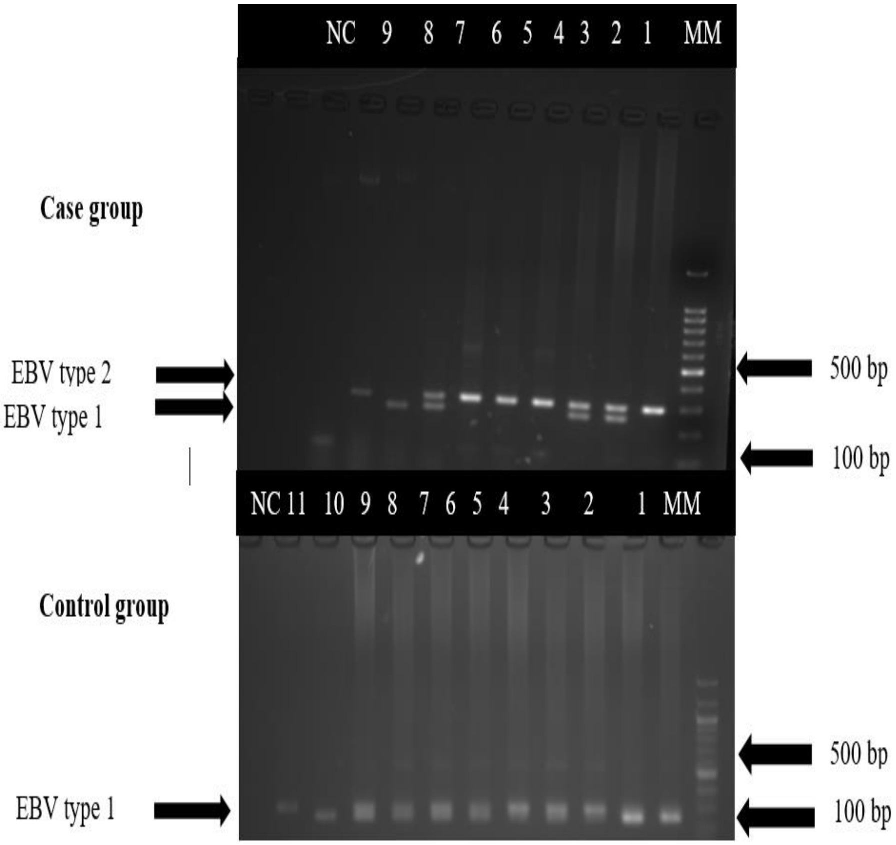

**Figure 4. 5. Detection of EBV genotypes in cases and control groups. Case group**: lanes 1 to 7, were EBV genotypes. Band sizes of 250 bp and 300 bp characterized genotypes 1 and 2, respectively. Lanes 8 and 9 were positive controls for types 1 and 2, respectively. **Control group**: lanes 1 to 8, were EBV genotypes. Band sizes of 250 bp and 300 bp characterized genotypes 1 and 2, respectively. Lanes 9 and 10 were positive controls for types 1 and 2, respectively. MM: 100 bp molecular weight marker, **NC**: negative control

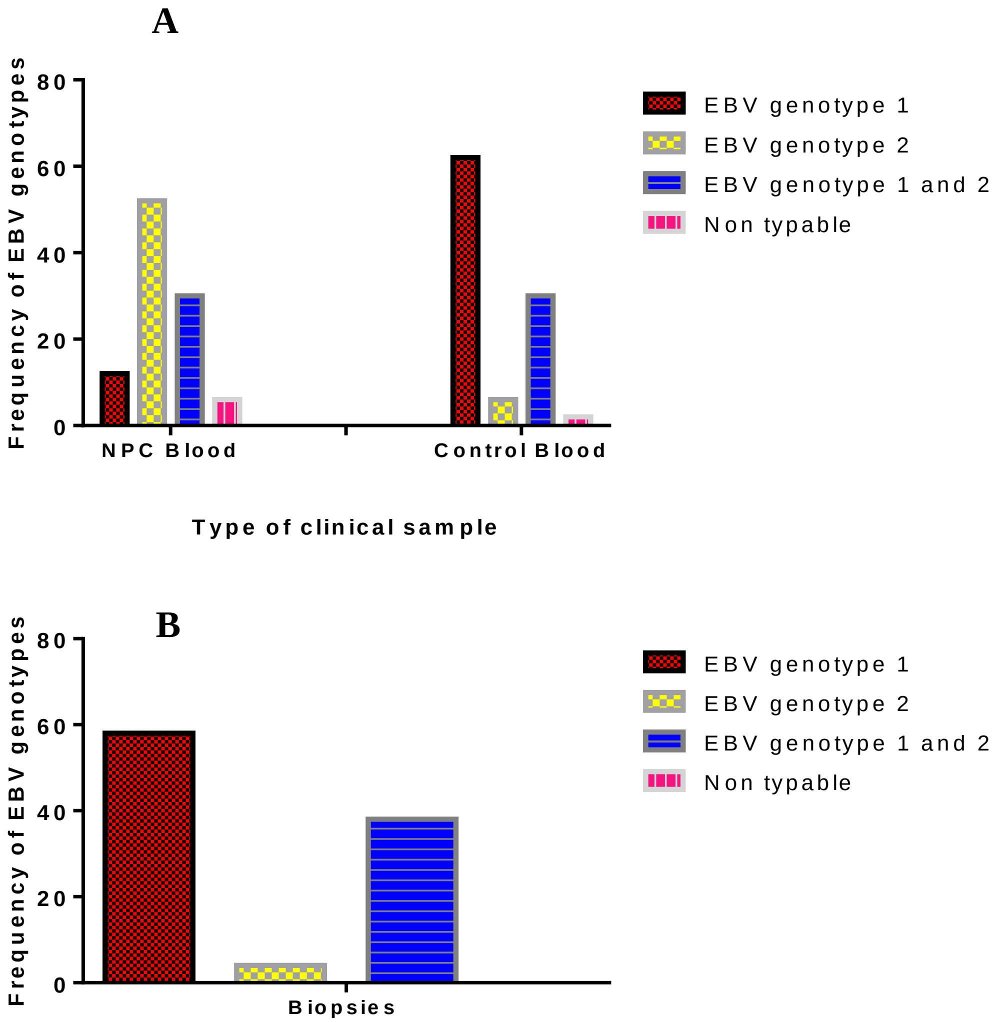

**Figure 4. 6. Frequency distribution of EBV genotypes in cases and control samples**

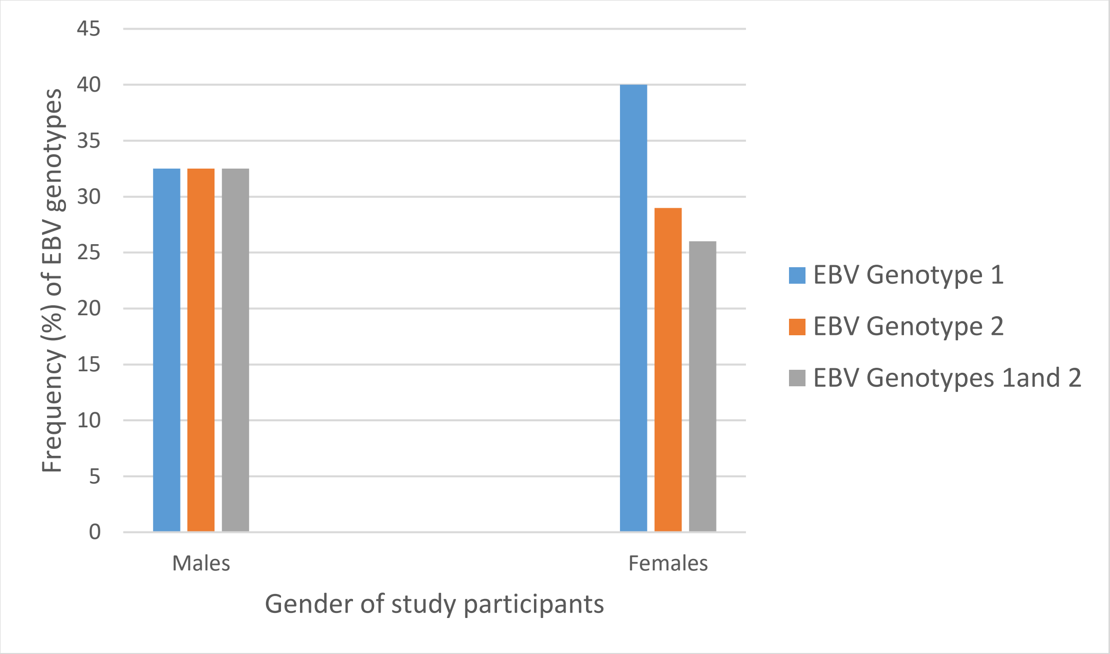

**Figure 4. 7. EBV genotype distribution among male and female participants**

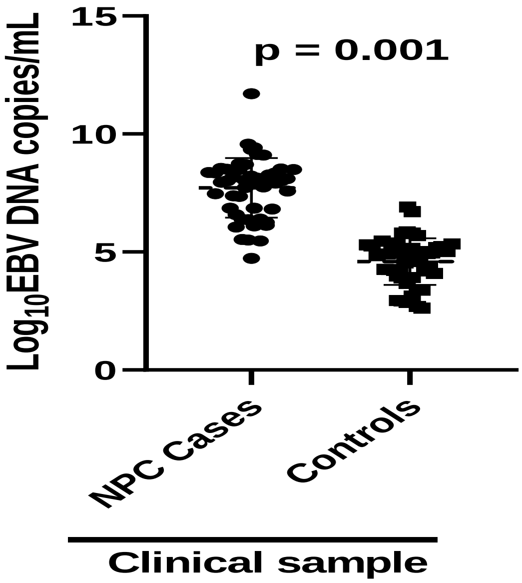

**Figure 4. 8**. **Comparison of EBV DNA load (copies/mL) in whole blood samples**. Epstein Barr virus (EBV) load quantification in whole blood samples was done by EBNA-1 real-time PCR in nasopharyngeal cancer (NPC) patients and healthy controls. EBV copies are represented on logarithmic scale ($Log_{10}$). Comparison of the median EBV load between cases and controls was done using Mann-Whitney test.

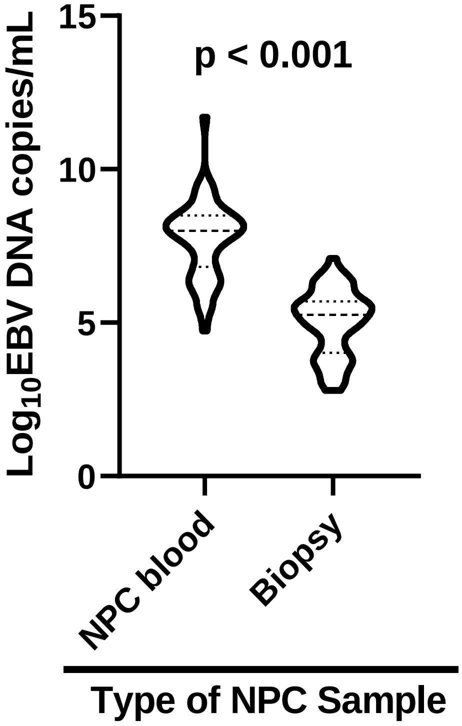

**Figure 4. 9**. **Comparison of EBV load in blood with biopsy specimen from NPC patients**. The median EBV DNA values from the samples were compared using Mann-Whitney test. `

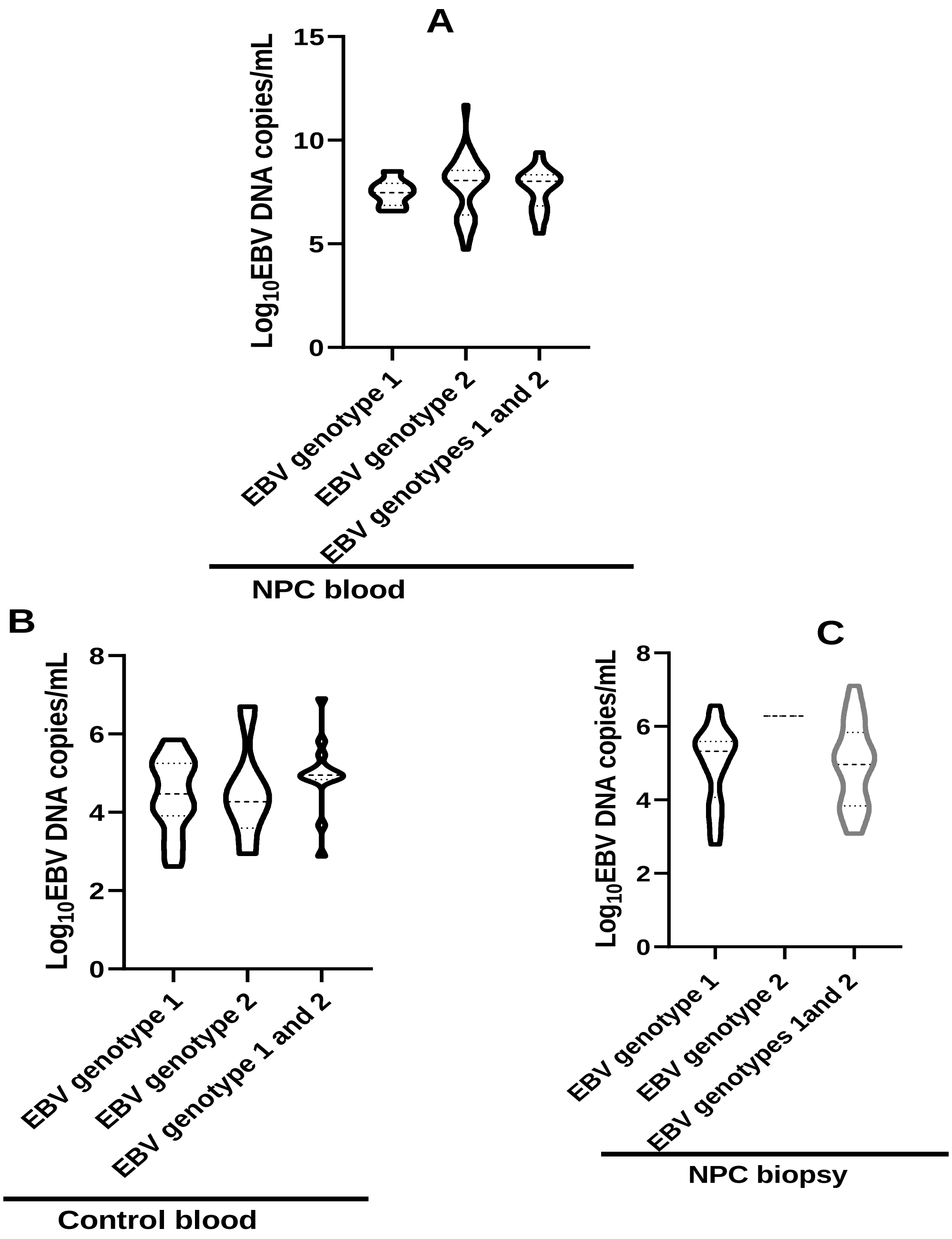

**Figure 4. 10**. **Within sample comparison of EBV load. Comparison of EBV genotypes viral load**. **(A) Viral load of EBV genotypes in whole blood samples from NPC patients. (B) Viral load of EBV genotypes in whole blood samples from healthy controls. (C) Viral load of EBV genotypes from biopsies of NPC patients.** Kruskal-Wallis test was used to compare median viral load of mono infections (EBV genotypes 1 or 2) and coinfections (EBV genotypes 1 and 2) in the respective samples. Comparison of the median viral loads showed no statistical significance ($p > 0.05$).

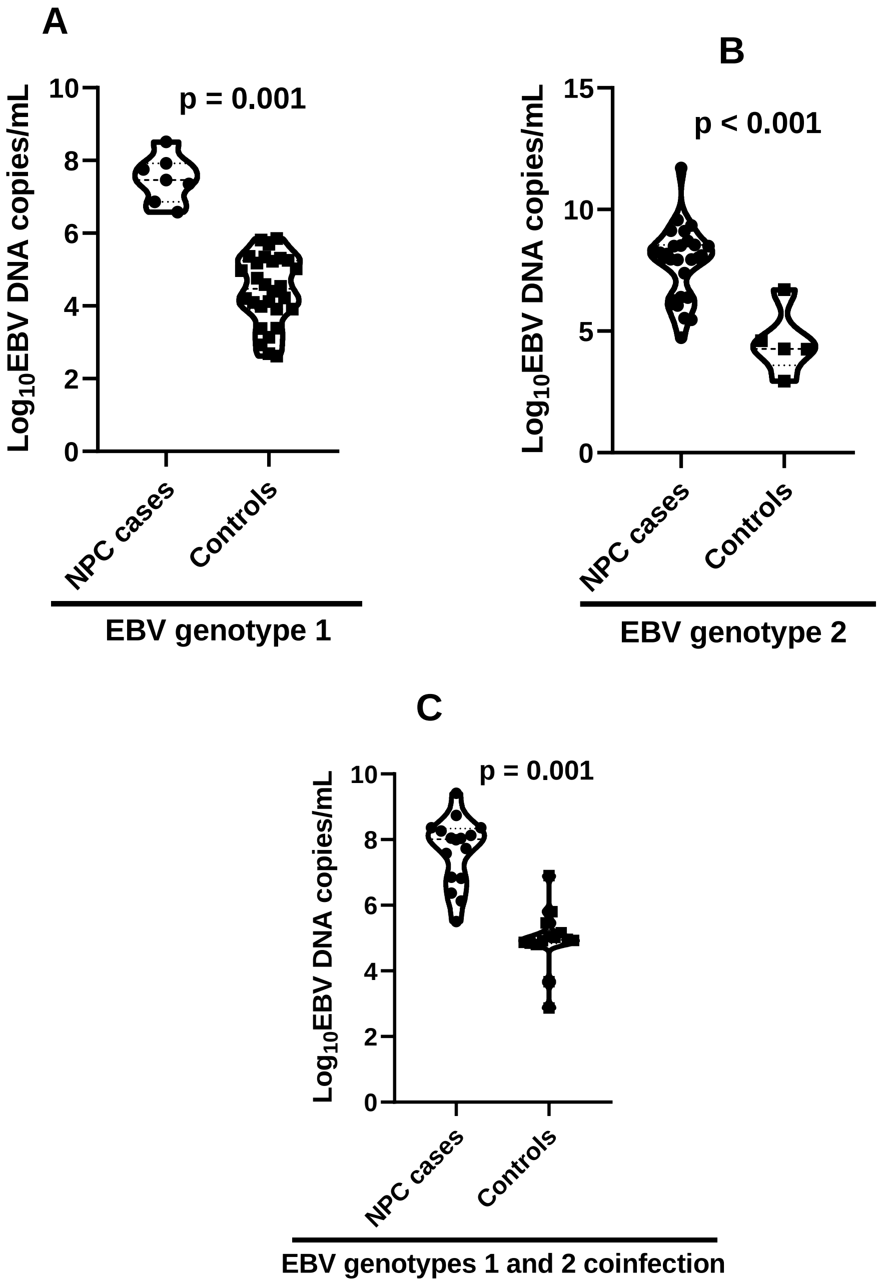

**Figure 4. 11. Comparison of EBV load between NPC patients and healthy control blood samples. (A) Median viral load of EBV genotype 1. (B) Median viral load of EBV genotype 2. (C) Median viral load of EBV genotypes 1 and 2 coinfecction**. Mann-Whitney test was used to determine difference in median viral DNA load. All genotype viral loads were significantly elevated in patients than healthy controls ($p < 0.05$)

# CHAPTER FIVE

## 5.0 Discussion

Nasopharyngeal cancer (NPC) is uncommon malignancy of the head and neck in most part of the world except for a few countries with Mongoloid origin (Borthakur *et al.*, 2016). Nasopharyngeal cancer has an incidence of 1 person per 100 000 in non-endemic regions in world, however, in endemic regions like Southern China and Asia the incidence ranges between 25 to 30 persons per 100 000 (Breda *et al.*, 2010). The etiology of the disease is multifactorial, involving a complex interplay of environmental, viral, genetic and dietary factors. Among these factors, NPC etiology is commonly associated with a viral factor known as Epstein Barr virus, a ubiquitous virus which infects more than 90% of the world's population. Association between NPC and EBV infection has been suggested due to the presence of the viral genome in NPC tumor biopsies and elevated antibody titers against EBV antigens in the sera of NPC patients (Banko *et al.*, 2016; Yao *et al.*, 2017). Two genotypes of the virus, namely EBV genotypes 1 and 2, exist and exhibit variation in geographical distribution. EBV type 1 is found globally but predominant in American, Chinese, European and South-East Asian (SEA) populations whereas type 2 is predominantly found in Africa (Peh *et al.*, 2002). The study therefore sought to detect and characterize EBV genotypes involved in NPC cases in Ghana and to also investigate association between the genotypes and the viral load in whole blood of these NPC patients.

## 5.1 Demographic Parameters and Regional Distribution of NPC Cases

In this study 55 patients diagnosed of NPC by endoscopy and CT scan at the Ear, Nose and Throat Unit of the Korle-Bu Teaching Hospital were enrolled. The median age of these patients was 40 years and this finding was in agreement with studies that reported the incidence of NPC being common among patients around the age of 40 (Guo *et al.*, 2009; Xiao *et al.*, 2013). For debatable reasons, NPC is predominant in males than females, with male to female ratio of 2-3:1 (Aziz *et al.*, 2017; Chang *et al.*, 2006; Mimi *et al.*, 2002). Some previous studies have attributed the variation in gender distribution of NPC to intrinsic exposures such as sex hormones and also environmental risk factors such as smoking and hazardous occupational exposures (Long *et al.*, 2017; Xie *et al.*, 2013). In the current study, there was an even distribution (1:1 male to female ratio) of NPC frequency in both sexes and this observation was in contrast to previous findings that reported male dominance. The uniform distribution of NPC in both males and females in this study could also be attributed to the fact that Ghana is a non-endemic region for NPC, and both sexes have equal chances for the development of the cancer (Table 1).

Nasopharyngeal cancer incidence is high in early adults (between 30 to 50 years) and declines in late adults (patients above 50 years) (Guo *et al.*, 2009). To evaluate this observation, NPC patients in this study were divided into 30-year age groups and it was observed that 44 % of the subjects were within 30 to 60 years, similar to previous reports (Amusa *et al.*, 2004; Nwawolo *et al.*, 2001). Findings previously reported by a group of researchers from Ghana, indicated that the incidence of NPC was highest among patients between the ages of 10-19 years (Larsen-Reindorf *et al.*, 2014), contrary to what was observed in the current study (Figure 4.2)

Also, regional distribution of NPC cases in this study, showed variation in the number of reported cases. The highest number of NPC cases were reported from the Eastern region of Ghana with the lowest cases originating from Northern, Upper East and Ashanti regions of Ghana. It has been shown that the frequency of NPC among individuals coming from coastal belt is very high due to constant exposure to salted fish, a potential risk factor of NPC (Kitcher *et al.*, 2004). This observation is contrary to what was seen, since most of the NPC cases were coming from the Eastern region of Ghana, which is not a coastal belt. The variation in the reported cases may be due to proximity of health facility, because Eastern and Volta regions are not too far from Korle-Bu Teaching Hospital, situated in Accra, as compared to Northern, Upper East and Ashanti regions (Figure 4.1).

## 5.2 Risk Factors Associated to NPC Development

The etiology of NPC is multifactorial, involving viral, genetic and environmental factors such as alcohol intake, smoking and consumption of salted fish. In this study, the association of NPC to the various risk factors were investigated.

Studies have reported the association of NPC to gender, with men having the highest incidence of the cancer as compared to women (OuYang *et al.*, 2015a; Umar *et al.*, 2014). However, in the current study, no association was established between NPC and gender. This observation may mean that, in non-endemic regions like Ghana, NPC is not gender associated. No association was observed between NPC and alcohol intake and this finding did not agree with previous reports which showed that consumption of alcohol increases the risk of cancer development in oral cavity, upper and lower pharynx (Lourembam *et al.*, 2015; Terrell *et al.*, 1999). However, the finding agreed with published reports by Guo *et al.* (2009), who stated that alcohol intake was not associated to NPC development (Guo *et al.*, 2009). Also, whiles smoking has been strongly associated to the development of NPC in some studies (Ji *et al.*, 2011; Long *et al.*, 2017), no link was established between smoking and NPC development in this current study. The finding from this study was supported by a previous report which showed that smoking was an independent risk factor for the development of NPC (Guo *et al.*, 2009). It could be deduced from the current study that, in non-endemic regions for NPC, smoking may not be the main cause of the cancer.

Unlike gender, smoking and alcohol, a strong association of NPC with regular consumption of salt-preserved fish was observed in this study. This finding agreed with previous reports where salt-preserved fish consumption increased the risk of NPC development in patients living in endemic regions (Ning *et al.*, 1990; Sriamporn *et al.*, 1992). However, finding from this study conflicted with reports from other parts of the world where no association was established between NPC and salt-preserved fish consumption (Hsu *et al.*, 2012; Lau *et al.*, 2013) (Tables 4.2 and 4.3).

### 5.3 Prevalence of EBV and Risk of Infection by Gender

Epstein Barr virus infects more than 90% of the world's population and most of the infections are asymptomatic (Breda *et al.*, 2010). The replication of the virus is held in check by EBV-specific cytotoxic T cells in immunocompetent individuals. However, in instances where immune surveillance by cytotoxic T cells wanes down, the virus is held out of check and this may result in various EBV associated cancers (Hatton *et al.*, 2014). In the host, EBV expresses restricted sets of latency genes that vary from tumor to tumor, however one of the latency genes which is expressed in all tumors is EBNA-1. Expression of EBNA-1 has also been found in asymptomatic carriers of EBV (Salahuddin *et al.*, 2018). In the current study, EBV was detected by amplification of EBNA-1 in whole blood and tumor biopsies of NPC patients and from the whole blood of individuals negative for any known oncological diseases (controls subjects). The highest frequency of EBV detected in blood samples of control participants as compared to blood samples from NPC patients confirmed the finding that about 90 % of the world's population are asymptomatic carriers of the virus. However the result obtained from the current was inconsistent with results reported from similar research conducted by Breda *et al.* (2010) in a non-endemic region like Ghana. In their studies, the frequency of EBV positivity was significantly higher in blood samples from NPC patients than control participants (Breda *et al.*, 2010). Also, a major public health implication that may be associated with the high frequency of EBV in blood samples of healthy subjects in the current study, is the risk of EBV transfer through blood transfusion, which has been reported in some studies (Alfieri *et al.*, 1996; Trottier *et al.*, 2012). The detection of EBV in biopsy specimen in this study was consistent with previous studies where EBV DNA was detected in tumor specimen of NPC patients (Klemenc *et al.*, 2006; Wang *et al.*, 2016) (Figure 4.4).

In the current study, association between EBV infection and gender was evaluated. It was observed that males and females had equal chances of getting EBV infection. The finding contradicts a recent research conducted in Morocco by El-Amrani-Joutey *et al.* (2018), where it was observed that the frequency of EBV infection was higher in males as compared to females (El-Amrani-Joutey *et al.*, 2018) (Table 4.4).

### 5.4 Distribution of EBV Genotypes in Blood and Biopsy Specimen

Epstein Barr virus can be classified into two main geographically distinct genotypes, A and B, or types 1 and 2, based on sequence polymorphism in the nuclear proteins, Epstein Barr Nuclear Antigen (EBNA-2) [(Banko *et al.*, 2016) ]. EBV genotyping in different specimen such as blood, saliva and tumor samples has been reported from different parts of the globe, however, to be the best of knowledge, limited studies have been done in Ghana to detect circulating EBV genotypes. A recent study conducted in Ghana by Asante *et al.* (2017), only reported the frequency of EBV in NPC biopsies. Therefore, the current study is the first EBV genotyping study in both NPC patients and healthy individuals in Ghana.

In the current study, EBNA-2 was used to genotype EBV in blood and biopsy specimen, to provide a snapshot of the genotype profile in these various specimen. It has been reported that the two EBV genotypes occur worldwide but vary in geographical distribution; EBV type 1 is found globally but is predominant in American, Chinese, European and South-East Asian (SEA) populations whereas type 2 is predominantly found in Africa (Peh *et al.*, 2002). However, in the present study, discrepancy in genotype distribution was observed. The predominant genotype of EBV in blood samples from NPC patients was EBV genotype 2 and that of control blood sample was genotype 1. Similar studies conducted in EBV genotype 1 predominant regions in the world, using blood specimen from both NPC patients and control subjects showed uniformity in genotype distribution. For instance, a study conducted by Klemenc *et al.* (2006) in Slovenia, a genotype 1 predominant region, using blood specimen from both NPC patients and healthy participants, showed the predominance of EBV genotype 1 in blood specimen from both study participants (Klemenc *et al.*, 2006). The prevalence of NPC is consistent with the geographical distribution of EBV genotypes. In NPC endemic regions like South-East Asia and China, EBV genotype 1 is predominantly found in specimen collected form NPC patients, suggesting a link between NPC and EBV genotype 1 (Banko *et al.*, 2016; Sung *et al.*, 1998). From the current finding, it could be deduced that the two genotypes are implicated in NPC cases in Ghana (Figure 4.6 A).

EBV belongs to the family *Herpesviridae* and a common feature associated with members from this family is multiple or co-infection in both healthy and diseased individuals (Beyari *et al.*, 2003; Meyer-König *et al.*, 1998). In the present study EBV genotypes 1 and 2 co-infection was detected in blood samples from both NPC patients and healthy subjects, consistent with findings reported from different parts of the world (Klemenc *et al.*, 2006; Kwok *et al.*, 2015). Sixbey *et al.* (1989) were the first to have reported EBV co-infection and subsequent reports demonstrated that in both immunocompetent and immunocompromised individuals, the two EBV genotypes could co-exist (Correa *et al.*, 2004; Sitki-Green *et al.*, 2004; Sixbey *et al.*, 1989). Due to sequence diversity in EBV, immune surveillance mounted by EBV specific cytotoxic T cell during primary infection does not confer preexisting host immunity against existing genotypes of EBV or future infections and as a result, multiple EBV genotypes could simultaneously exist in the host (Henry *et al.*, 2001). This explanation could account for the detection of co-infection in both NPC patients and the healthy subjects in the present study (Figure 4.6A).

EBV genotype profiling was carried out in tumor biopsies from NPC patients in the present study and it was observed that the predominant genotype is EBV genotype 1. The observation from this study mirrored the frequency observed in NPC-EBV type 1 endemic regions. For instance, in NPC endemic regions like Chinese, European and South-East Asian (SEA) populations, EBV genotype 1 is frequently identified in NPC tumor biopsies (Banko *et al.*, 2016; Cui *et al.*, 2011; Ikegaya *et al.*, 2008). It has been reported that EBV type 1 has high tumorigenic potential than EBV type 2 (Sung *et al.*, 1998), and this finding may account for the prevalence of the former than the latter in the present study. One should have expected the predominance of EBV genotype 2 in the biopsies since Africa is predominated by this genotype. However, a different pattern was observed in the present study. The finding from this study is supported by a previous study in Africa where EBV genotype 1 was predominant in tumor biopsies collected from NPC patients (Bouzid *et al.*, 1994). Similar to blood samples from the NPC patients, EBV genotypes 1 and 2 coexisted in the tumor biopsies, supporting the finding that primary infection of one EBV genotype does not confer immunity against future infection of a different EBV genotype (Henry *et al.*, 2001) (Figure 4.6 B).

## 5.5 Distribution of EBV Genotypes by Gender

The present study has evaluated the frequency of EBV genotypes among male and female subjects and it was observed that there was homogeneity in genotype distribution in male subjects. However, in female participants EBV genotype 1 was predominant. This finding was inconsistent with a recent study by Salahuddin *et al.* (2018) , who reported that there was no variation in distribution of EBV genotypes among male and female subjects (Salahuddin *et al.*, 2018). Evidence from a cohort study by Higgins *et al.* (2007) who investigated the distribution of EBV genotypes among students in University of Edinburgh, indicated that EBV genotype 1 was predominant among female than male students (Higgins *et al.*, 2007). The finding from this study was consistent with finding from the current study showing the predominance of genotype 1 in Ghanaian female subjects (Figure 4.7).

### 5.6 Epstein Barr Viral Load Levels among NPC Patients and Healthy Control Subjects.

Limited studies have reported EBV DNA load in whole blood or biopsy specimen. Therefore, in the current study, for the first time in Ghana, EBV DNA load was quantified in whole blood and biopsy samples from NPC patients and healthy control participants. Comparison of whole blood EBV DNA load between NPC patients and healthy control participants was done to determine statistical difference in viral burden among the two study populations. Unfractionated whole blood samples were used because previous report had demonstrated that EBV DNA quantification from unfractionated whole blood combines all blood components that may host the virus and serves as a good reporter of the absolute viral burden in the patient’s circulation (Stevens *et al.*, 2001). The higher viral load observed in whole blood from NPC patients compared to healthy controls was consistent with findings reported by researchers from other parts of the world. For instance, a case-control study to determine the EBV DNA load in whole blood samples from 105 NPC patients and 115 healthy controls in India by Lourembam *et al.* (2015) has shown that, significantly high viral load was present in NPC cases compared to controls (Lourembam *et al.*, 2015). Another study by Adham *et al.* (2013) involving whole blood samples from NPC cases and control subjects showed significantly high EBV DNA load in cases compared to controls (Adham *et al.*, 2013). After primary infection, EBV establishes latent infection in memory B cells at a low viral level (about 1 in 10,000 to 100 000 B cells), hence, healthy individuals may carry quantifiable EBV loads in their circulation but in low levels (Thorley-Lawson *et al.*, 2004). However, in NPC patients, blood EBV DNA level is derived from cancerous epithelia tumor cells and also circulating B cells (Chan *et al.*, 2003). EBV DNA from these two sources of cells in NPC patients may account for the significantly high viral load when compared to the healthy subjects in the present study. Data from the current study suggested that pretreatment assessment of EBV load in whole blood could serve as a useful supplement for diagnosis of NPC in non-NPC endemic regions like Ghana, instead of tumor biopsy which is invasive and difficult to access (Figure 4.8).

The current study also sought to evaluate the distribution of EBV DNA load in different clinical specimen from NPC patients. To the best of knowledge this is the first study to have reported the distribution of EBV load in NPC whole blood and tumor biopsy specimen. Significantly high EBV DNA load was observed in NPC whole blood specimen compared to the tumor biopsy. In EBV associated malignancies there is active replication of the virus in tumor cells, hence EBV maintains high level of viral load in circulation and to achieve this, more than 100 million copies of the viral DNA must be released in the circulation daily (Chan, 2014; Fung *et al.*, 2016). Therefore, high viral load in NPC whole blood samples compared to that of tumor biopsy in the present study may be explained in light of the aforementioned finding. Moreover, EBV DNA in whole blood samples from NPC patients originates from B cells, tumors cells and unnatural targets such as T cells and natural killer cells, hence, may account for the variation in viral load distribution between NPC blood and tumor biopsies observed in the current study. Also, from this study, it could be deduced that pretreatment viral load quantification in the whole blood of NPC patients may be a non-invasive procedure for predicting tumor burden, diagnosis and determining the outcome of chemotherapy (Figure 4.9)

Study participants in the current research had mono infection of EBV genotypes 1 or 2 with some having co-infection of the two EBV genotypes. The study sought to evaluate intra-sample dynamics of viral load distribution in each type of clinical specimen harboring different genotypes of EBV. This idea was imported from a previous study done by Petrara *et al.* (2014), to investigated the distribution of EBV DNA load in HIV infected children in Uganda, with mono or co-infection of EBV genotypes. These researchers found out that EBV DNA load was higher in patients co-infected with the two genotypes of EBV compared to patients with either EBV genotype 1 or 2 mono infections (Petrara *et al.*, 2014). However, in the present study, no statistical difference was observed in the distribution of EBV DNA load in clinical samples having different profile of EBV genotype. The data from this study may also suggest that despite the predominance of EBV genotype 1 in control subjects or genotype 2 in NPC patients, viral level may not be influenced by predominant genotype as observed in other viruses such as Hepatitis B virus (HBV), where HBV genotype C was associated to increased viral load in patients with hepatocellular carcinoma (HCC) (Yu *et al.*, 2005) (Figure 4.10)

Case-control genotype specific EBV DNA load comparison data in this study indicated that NPC patients with mono or co-infection of EBV genotypes 1 and 2 had significantly high whole blood viral load compared to that of control subjects. A possible reason for the difference may be due to the contribution of EBV DNA from tumor cells to the pool of whole blood EBV DNA in NPC patients (Figure 4.11).

# CHAPTER SIX

## 6.1 CONCLUSIONS

This study detected and characterized EBV genotypes 1 and 2 in both blood and tumor biopsies form study participants in Ghana. Epstein Barr virus DNA frequency was higher in control participants than that of cases, confirming reports suggesting large number of the world's population being asymptomatic carriers. The predominant EBV genotypes in whole blood samples from control subjects was EBV genotype 1 and that of NPC blood and biopsies were genotypes 2 and 1, respectively. Whole blood sample from NPC patients had higher pretreatment median viral load compared to pretreatment median viral load in whole blood of control participants and this suggests that EBV load quantification can be used as a non-invasive biomarker for NPC diagnosis in Ghana.

## 6.2 LIMITATIONS

Despite the above findings, the results from the current study should be explained with the consideration of some limitations. Limitations to this study are the relatively smaller sample size used, and the unavailability of histopathological results to confirm the NPC status of the patients.

## 6.3 RECOMMENDATION

Based in this study, immunohistochemistry studies must be done on the tumor biopsies to identify biomarkers confirming the presence of NPC.

Also, a follow-up study should be done to associate virulent factors with the EBV genotypes, and possibly suggest a mechanistic role of the factors in disease etiology, which will ultimately drive the search for therapeutic targets.

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# APPENDICES

## APPENDIX I

## NMIMR-IRB CONSENT FORM

**Title: Detection and Characterization of Epstein Barr Virus in Nasopharyngeal Cancers in Ghana**

**Principal Investigator: Richmond Ayee**

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana. P. O. Box LG 54 Volta Road Legon.

### General Information about Research

Nasopharyngeal cancer (NPC) is a type of cancer which occurs in the nasopharynx (inside the throat) and it is caused by several factors. One of these factors is a virus known as Esptein Barr virus (EBV).Association between NPC and Epstein Barr virus (EBV) (cancer causing virus) has been reported due to the presence of high level of antibodies against EBV as well as the detection of EBV DNA in the blood of infected individuals. Different types of EBV have been reported to be associated with NPC and these EBV types vary from one region to the other. There is no information in literature concerning the types of EBV involved in NPC cases in Ghana. Therefore, this study is aimed at detecting and characterizing EBV types involved in the development of NPC in Ghana. The outcome of this study will provide insight into the existing EBV types and their role in NPC development in Ghana. The study will also provide information that will be useful in the development of EBV vaccines in the future. The study will involve the collection of cancer tissues, blood and saliva samples from patients diagnosed of NPC as well saliva and blood samples from healthy individuals. The samples will be collected after the subjects have agreed and the whole procedure will take about 30 minutes.

### Possible Risks and Discomforts

The needle may hurt, there will be some bruising and pain at the site of pricking. This will be done by a qualified personnel (Dr. Kafui, ENT specialist), and therefore significantly minimizes the risk of pain and infection

### Possible Benefits

The diagnosis of NPC is mostly difficult due to non-specific symptoms and this results in the recognition of the disease only after the development of the cancer. Therefore, the outcome of this study will provide insight into existing EBV types and their role in NPC development in Ghana. This can provide information that will be useful in the development of EBV vaccines in the future.

### Confidentiality

We will protect information about you to the best of our ability. The information of your medical records will be protected. Information that identifies you, such as consent form and questionnaire will be kept under locked and secured. If information from the research is published or presented at scientific meetings, your identifiers will not be included. Information about you will be destroyed after research is completed. You will not be named in any report. The supervisor of the study is the only person who may take a look at your medical records.

### Compensation

There will be no compensation, be it monetary or any form, given to you for your participation in this study.

### Additional Cost

There is not going to be any additional cost to you for participating in the research.

### This session does not apply to all studies

Voluntary Participation and Right to Leave the Research

This research is purely voluntary and you can withdraw from the study without any penalty and compensation to the researchers involved

## Contacts for Additional Information

Dr. Osbourne Quaye, PhD; Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon. Tel: +233-277-459566. Email: OQuaye@ug.edu.gh

## Your rights as a participant

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.ug.edu.gh

## VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title “Detection and Characterization of Epstein Barr Virus in Nasopharyngeal Cancers in Ghana” has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

_______________________ _________________________________________________

Date Name and signature or mark of volunteer

**If volunteers cannot read the form themselves, a witness must sign here:**

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

_________ ________________________________________

Date Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

________________________ ________________________

Date Name Signature of Person Who Obtained Consent

# Questionnaire

## Detection and Characterization of Epstein Barr Virus in Nasopharyngeal Cancers in Ghana

Type of specimen: ☐Blood ☐Saliva ☐Tissue biopsy

Date specimen collected:………../………./………

## Patient Information

Patient #...................Last name: …………………………………….

First name: …………………………………………………

Names of Parents/ Guardian (if child):

Father…………………………………………..
Mother………………………………………..

Hometown of parents: Father…………………………………..
Mother…………………………..

Location of birth………………………………………… Most time spent
……………………….

Type of location (town or village)……………………………………………

Settlement………………………………. Region…………………………..………..

Occupation…………………………………………………………………………….

Educational level (Primary/ Secondary/
University)…………………………………………………

DOB………../…………. /…………. Age ………. (Years) Sex: ☐ Male ☐ Female

**Social habits:**

**Alcohol** ☐ Yes ☐ No **Smoking** ☐ Yes ☐ No **Eating of salted fish** ☐ Yes ☐ No

## APPENDIX II

### Sample size calculation

$n=z^2 \times p \times (1-p)/\varepsilon 2$

Where n is sample size

P is is the frequency of NPC = 1.3%

$\alpha = 0.05$

$\varepsilon$ = margin of error (0.06)

## APPENDIX III

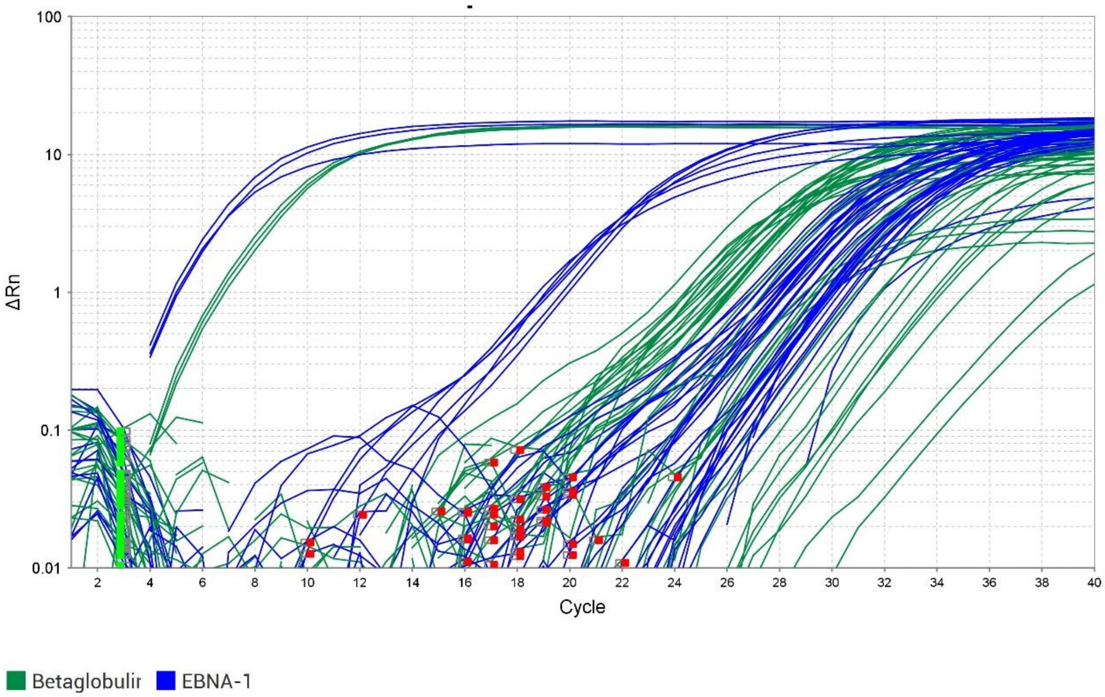

Figure 4.11: **Amplification plot for EBV load quantification by real-time PCR**. Viral load quantification was done by amplification of EBNA-1 (target gene) and normalized by co-amplification of betaglobin.

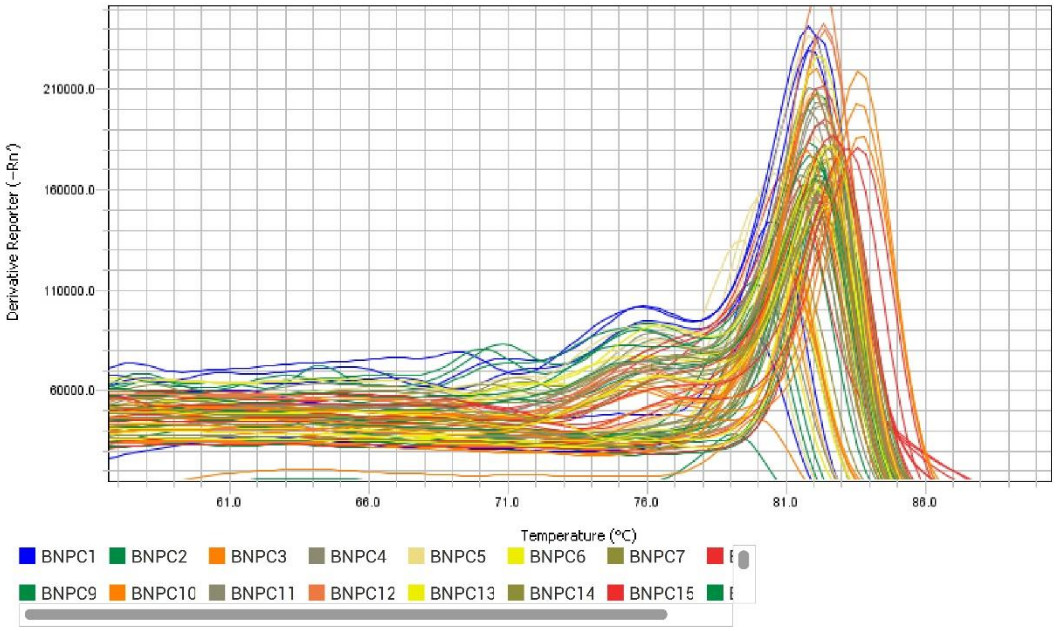

Figure 4.12: **Melt curve plot**. Specificity of target amplification was confirmed by melt curve analysis over temperature range of 78-85 ºC