CHAPTER 1

INTRODUCTION

1.1   General Introduction

Nasopharyngeal carcinoma (NPC) is the malignancy of epithelial cells in the post-nasal space of the nasopharynx. The most common site of origin of NPC is the fossa of Rosenmuller (Prasad *et al.*, 1983; Yew, 1991). NPC occurs at high frequencies (10-30 new cases per year/100 000) in Chinese populations: mainly those living in the Southern provinces of the Republic of China, Hong Kong, Chinese emigrants in South-East Asia and other countries. NPC is also prevalent among Eskimos in Greenland and Alaska but rare in Europe and America (incidence below 2 new cases per year/ 100 000). Intermediate rates are seen in North and East Africans and Malays living in Malaysia or in Singapore (Simons & Shanmugaratnam, 1982; de The & Zeng, 1986). It affects a younger age group population compared to any other head and neck neoplasm, with at least 60% of patients being under 50 years of age (Skinner *et al.*, 1991).

The symptoms of NPC vary tremendously from one patient to another, causing delay or misdiagnosis, additionally, clinical symptoms of NPC appear late after the onset of malignancy. The delay or misdiagnosis causes high mortality among NPC patients. Woo & Waldron (1991) developed a multiple approach for the detection of NPC. Biopsy is used as the basis of diagnosis of patients with obvious nasopharyngeal abnormality, while patients suspected clinically as having NPC but with normal-looking nasopharynx, several procedures are recommended. These include deep

biopsies from multiple sites, fine needle aspiration cytology, imaging test, radiology test and detection of Epstein-Barr virus (EBV) antigens and antibodies. Clinicians often face difficulties visualising the tumour and obtaining representative biopsy specimens for histology (Prasad *et al.*, 1983; Woo & Waldron, 1991), hence, a combination of clinical examination and laboratory investigation is a valuable aid to the clinician in the diagnosis of NPC.

Malaysia comprises the Peninsular Malaysia (West Malaysia), Sabah and Sarawak (East Malaysia). The Peninsular is situated south of Thailand while Sabah and Sarawak occupy northern Borneo. Sabah and Sarawak are separated from the Peninsular by the South China Sea (Figure 1.1). The outstanding characteristic of the Malaysian population is its highly variegated ethnic mix. Malaysians consist of various ethnic groups such as Malay (51.2%), Chinese (26.9%), Indians (7.7%) and other indigenous groups (10.9%) and others (3.3%). The Malays form the predominant ethnic group in Peninsular Malaysia (59.0%). In Sabah, the Kadazan (23.9%) form the largest ethnic group with the Murut, Kelabit and Kedayan forming significant minorities. In Sarawak, the Ibans (29.6%) form the largest ethnic group in the state (Information Malaysia, 2000).

Much of the work on NPC in Malaysia has been done on West Malaysian patients. Since NPC has both genetic and environmental causes, the role of EBV as an environmental factor may be different in different population of ethnic origins. The different composition of ethnic groups in the West and East may result in differences in the frequency of NPC.
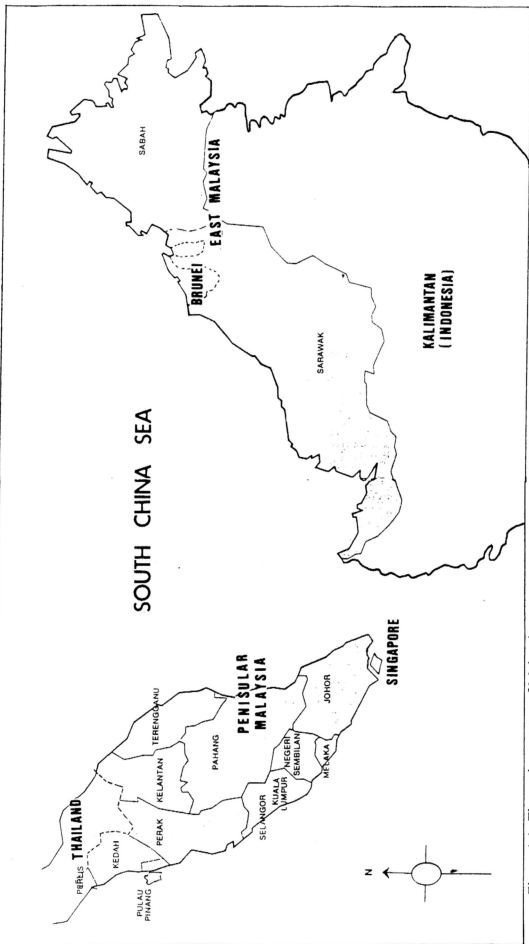
Figure 1.1: The various states of Malaysia

1.2   Ethnic Groups of Sarawak

Sarawak is one of the two states of East Malaysia and is the largest state according to area in Malaysia. It is situated in the northern part of the island of Borneo and shares its southern boundary with Kalimantan, Indonesia. To the northwest, Sarawak adjoins the other East Malaysian state Sabah and the Sultanate of Brunei Darulsalam (Figure 1.1).

In a preliminary estimation of the Department of Statistics, Malaysia, the total population of Sarawak in 1998 was 1.98 million. The ethnic composition in Sarawak comprised of Malay, Iban, Bidayuh, Melanau, other indigenous groups, Chinese and others. The population estimate by ethnic group is shown in Table 1.1. The Ibans form the largest ethnic group in the state (Information Malaysia, 2000)

Table 1.1: Population estimate by ethnic group

Ethnic group	Population ('000)	Population (%)
Malay	431	21.8
Iban	586	29.6
Bidayuh	165	8.3
Melanau	114	5.8
Other indigenous	122	6.2
Chinese	543	27.4
Others	18	0.9

1.3   Epidemiology of NPC

In Hong Kong, the age-adjusted incidence rate of NPC for 1988-1992 was 24.3 per 100,000 for males, making it the 3rd most common cancer after lung and liver cancers. In females, it was the 8th most frequent cancer with the incidence rate of 9.5 per 100,000. For the same period in Singapore, NPC was reported to be the 4th and 8th most common cancer in Chinese males and females respectively, with incidence rates of 18.5 and 7.3 per 100,000 (Parkin *et al.*, 1997).

In Malaysia, due to the absence of an effective National Cancer Registry, comprehensive information about the distribution and incidences of cancer is still not available. However, NPC is among the most common cancers, especially in ethnic Chinese. For the year 1988, the overall age-adjusted incidence rate of NPC in Peninsular Malaysia was 3.2. Patients of Chinese ethnic origin made up of 86.3% of NPC patients followed by 12.9% Malays and 0.8% Indian. For the same year, the Chinese Malaysian had the highest age-adjusted incidence rates of 40.1 per 10,000 for males and 14.9 for females for the age group of 40-49 years, which is the age group with the highest incidence of NPC (Prasad & Rampal, 1992).

Norhanom (1989) in an eleven years (1975-1986) epidemiological studies on NPC incidence in Malaysia noted the highest annual incidence in the Chinese with 6.83 per 100,000. The Cantonese subethnic group comprised 34.3% followed by the Hokkiens at 27.0% and Hakka/Kheks at 23.1%. The Iban and Kadazan populations of East Malaysia also have high risk for NPC with incidence rates of 3.41 and 1.06 per 100,000. Low annual incidences of 0.73 and 0.21 per 100,000 were reported in Malays and Indians. In the same study, the age distribution pattern of Chinese and Ibans NPC peaks at the age of 40-44 years and 50-54 years for the Malays, however, in the Kadazans peaks were reported in age groups of 10-24 and 45-49 years.

1.4 Classification of NPC

NPC refers exclusively to carcinomas derived from the stratified squamous epithelium of the post-nasal space. The tumour is classified into histological types according to the degree of differentiation and to the extent of associated lymphoid stroma. There are 3 major classification systems, namely the French system (Micheau, 1978), World Health Organisation (WHO) Scheme (Shanmugaratnam & Sobin, 1978) and Cologne Scheme (Kruger & Wustrow, 1981).

The French classification recognises only two variants: squamous cell carcinoma (SSC) and undifferentiated carcinoma of nasopharyngeal type (UCNT).

NPC is classified according to the WHO classification into three variants:

i) Keratinizing squamous cell carcinoma (Type I, SSC) displays definite differentiation of squamous cells with the presence of intracellular bridges and/or keratinization. It can be further subdivided into well differentiated, moderately differentiated or poorly differentiated types. Type I SSC comprises 20% of NPC cases in Europe and U.S.A (Weiland, 1978).

ii) Non-keratinizing carcinoma (Type II, NKC) shows no squamous differentiation. The tumour cells have fairly well defined cell margins and show an arrangement that is stratified or pavemented and not syncytial. A plexiform pattern is present in many tumours. Some cells may exhibit a clear-cell structure, due to the presence of cytoplasmic glycogen.

iii) Undifferentiated (anaplastic) carcinoma (Type III, UC) generally has vesicular nuclei and prominent nucleoli. The cell margins are indistinct and the tumour exhibits a syncytial rather than a pavemented appearance. Tumour cells are arranged in irregular and moderately well defined masses and/or in strands of

loosely connected cells in a lymphoid stroma. In Malaysia, UC is the most common histological type, comprising 44% of NPC cases (Prathap *et al.*, 1983).

The Cologne classification is similar to the WHO scheme and in addition takes into consideration the importance of lymphoid stroma in the tumour tissue. The non-keratinizing carcinoma (NKC) and undifferentiated carcinoma (UC) are subdivided into without lymphoid stroma (Type a) and with lymphoid stroma (Type b).

Generally, WHO II and WHO III NPC occur in higher proportions of tumour in younger patients whereas WHO I NPC is rare, and found largely in older patients. WHO II and WHO III tumours respond better to therapy and patients have a longer disease-free period as compared to patients with WHO I tumour. The survival rates for WHO II and WHO III patients are also higher although generally survival rate is higher with earlier detection (Scanlon *et al.*, 1967; Dickson, 1981).

1.5   Aetiology Factors of NPC

The aetiology of malignancy is multifactorial and NPC is thought to be the result of genetic and environmental factors.

1.5.1   Genetic factors

The demographic patterns of NPC incidence suggest a genetic aetiology of the disease, however evidence for that has not been well established. The genetic association of NPC with the human leukocyte antigen (HLA) region on chromosome 6 especially at the alleles of the HLA loci A, B and DR was reported among the

Singaporean Chinese (Simons *et al*., 1973; Simons *et al*., 1974), Malaysian and Hong Kong NPC patients (Simons *et al*., 1977).

In Southern Chinese, the predominant HLA-haplotypes are i) A2-Cw11-Bw46 and DR9 and ii) Aw33-C3-Bw58 and DR3. Those people having HLA-A2 antigen have an increased risk of developing NPC, and A2 commonly occurs as the haplotype A2-Cw11-Bw46 without DR9 in Chinese NPC patients. This haplotype with the relative risk of 3.4 is associated with older onset patients (>30 years) and more common in long term survivors. The Aw33-C3-Bw58 /DR3 haplotype with the relative risk of 2.2 is associated with younger onset patients (<30 years). These patients have poor prognosis (Chan *et al*., 1983; Chan, 1990).

Lim & Chan (1996) reported that deletions around C4 and 21-hydroxylase (21-OH) gene situated at HLA Class II region occurs among Chinese NPC patients. The C4B together with 21-OHA deletion is the most common. However, the high frequency of this deletion in NPC was secondary to the B58 DR3 association with NPC as non-B58 haplotypes NPC patients do not have this deletion.

Besides the HLA associated gene, cytogenetic and molecular analysis had demonstrated consistent deletion of alleles on the short arm of chromosome 3 in NPC tumour tissue (Huang *et al*., 1989; 1991). Analysis of tumour DNA reported by Choi *et al*. (1993) showed NPC patients had a high frequency of loss of heterozygosity at two loci on chromosome 3 (3p25 and 3p14) suggesting that these deletions contribute to NPC. A consistent loss of constitutional heterozygosity within the specific chromosome locus is suggestive of a tumour suppressor gene. Such tumour suppressor gene, if deleted may encourage the development of tumours.

1.5.2 Environmental factors

Ho (1972) first proposed salted fish as a possible aetiological factor in the development of NPC in Chinese. Salted fish is a common food item among the Chinese populations (Ho, 1978), the Kadazans in East Malaysia (Rothwell, 1978) and other South-East Asian populations (Armstrong, 1983). All these groups have intermediate to high susceptibilities to NPC. Earlier epidemiological studies of excluding EBV and genetic factors may have resulted in an overestimation of salted fish (environmental factor) as an important aetiological factor (Chia & Lee, 1997). The salted fish contains volatile nitrosamines, principally N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosodi-n-propylamine and N-nitrosodi-n-butylamine (Huang *et al.*, 1981). Nitrosamines have been implicated as a nasopharyngeal carcinogen in humans. A study in Taiwan noted an excess of NPC cases with high activity of cytochrome P450 2E1 (CYP2E1), an enzyme that participates in the metabolic activation of nitrosamines *in vivo* (Hildesheim *et al.*, 1997).

A study by Armstrong *et al.* (1998) showed 4 salted preserved foods (fish, leafy vegetable, egg and root), pork/beef organ meats, beer and liquor consumption have clear positive association with NPC among Malaysian Chinese population. The increased risk of NPC was also linked to low consumption of foods rich in Vitamin A, C, E, beta carotene and/or phytochemicals. Other preserved foods e.g. fermented fish sauce, salted shrimp paste, salted soy beans, fermented bean curd, preserved plums, salted duck eggs, canned pickled vegetables, salted mustard greens 'kiam chye' and 'chung choi' have also been associated with NPC (Yu *et al.*, 1988; 1989; Ning *et al.*, 1990; Lee *et al.*, 1994).

Non-dietary factors such as tobacco, occupational exposure to fumes and chemicals (Yu *et al*., 1990), traditional chinese medicines (Li *et al*., 1985; Yu *et al*., 1988) are also thought to be linked to the development of NPC. A study in Shanghai found active cigarette smoking also contributing to the development of NPC (Yuan *et al*., 2000).

Another environmental factor is the Epstein-Barr virus (EBV). The association with NPC was first observed by Old *et al*. (1966) by the Ouchterlony double immunodiffusion of antibodies to various EBV-associated antigens from sera of NPC patients. This finding was confirmed by Henle *et al*. (1970a) using the immunofluorescence assay (IFA) technique and subsequently many other studies and vast literatures have shown evidences that NPC patients have higher EBV antibody titres than controls, regardless of geographical and ethnic origins (Henle *et al*., 1970a; Henle & Henle, 1985). Higher EBV antibody titres, particularly of the IgA class are characteristic of NPC and differ from those seen in healthy individuals (Henle & Henle, 1976). Additionally the EBV DNA is consistently found in NPC tumours (Wolf *et al*., 1973; Raab-Traub *et al*., 1987). The close association of EBV with NPC is an established fact, but the role of EBV in NPC remains to be determined, however EBV DNA and proteins are important markers of NPC.

1.6   Epstein-Barr Virus (EBV)

The Epstein-Barr virus (EBV) is also known as Human Herpes Virus 4 (HHV4) and is a gamma herpes virus in the Herpesviridae family (Figure 1.2). The virus was first observed under the electron microscope in a cell culture of an African Burkitt's lymphoma (Epstein *et al.*, 1964). EBV is closely related to viruses present in the old world nonhuman primates, including EBV like viruses of chimpanzees and rhesus monkeys. The rhesus monkey virus and EBV share similar sequences and genetic organisation (Moghaddam *et al.*, 1997). EBV is the etiologic agent of infectious mononucleosis (IM) (Henle *et al.*, 1968) and is closely associated with two human malignancies: African Burkitt's Lymphoma (BL) and NPC (zur Hausen *et al.*, 1970).

Similar to other herpes viruses, primary infection of EBV is followed by life-long carrier status. Approximately 95% of adults' world-wide carry the EBV (Epstein & Morgan, 1983; Henle & Henle, 1985). Most infections occur within the first years of life and are asymptomatic, occasionally associated with mild illness. In most developing countries, primary infection affects almost all children before the age of ten years and IM is almost unknown (Henle *et al.*, 1968). In privileged classes of developed societies, primary EBV infection is delayed until adolescence or adulthood. Approximately, 50% of these late primary infections result in the clinical syndrome of IM (Henle & Henle, 1973). EBV seroconversion is 100% by the time Malaysian children are 9 years old (Chua, 1999). In most nosocomial infections, EBV infects humans via salivary contact (Hoagland, 1955; Morgan *et al.*, 1979; Sumaya & Ench, 1986).
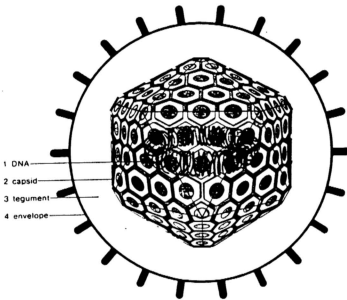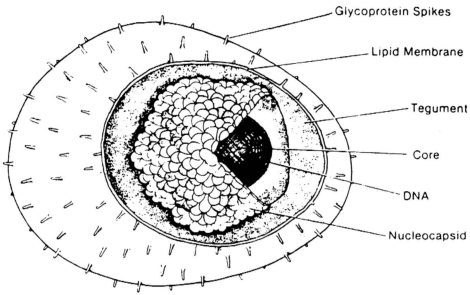
Figure 1.2: Herpesvirus structure and morphology.
(Ackermann & Berthiaume, 1995)

1.7   EBV Structure

An EBV particle is about 180 nm in diameter, with a toroid-shaped core protein wrapped with DNA, a nucleocapsid with 162 capsomeres, an outer envelope and a protein tegument between the nucleocapsid and the envelope (Epstein & Achong, 1973; Epstein *et al.*, 1965). The outer envelope consists of external high molecular-weight glycoprotein spikes (Edson & Thorley-Lawson, 1981) (Figure 1.2). The virus particle contains at least 33 different structural polypeptides. The major EBV capsid proteins are 160, 47 and 28 kD (Dolyniuk *et al.*, 1976). There are also a number of minor virion proteins and the most abundant EBV envelope and tegument proteins are 350/220 and 152 kD respectively (Mueller-Lantzsch *et al.*, 1980; Thorley-Lawson & Poodry, 1982).

There are two EBV types, namely types 1 and 2. The two types differ in only a few genes, but they make significant differences which are important in biological activity (Kieff, 1996). The differences occur in genes coding for the EBV nuclear antigens (EBNAs) 2, 3A, 3B and 3C with predicted primary amino acid sequence varying from 16 % to 47 % (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985; Sample *et al.*, 1990). Variation of ten single-base changes also appears in the genes of the small non-polyadenylated RNAs (EBERs) (Arrand *et al.*, 1989).

Type 1 EBV was found to be the predominant type in most southern Chinese (96%) or Taiwanese (94%) patients with NPC or other EBV-positive head and neck tumours. Type 2 (4%) or the coexistence of type 1 and 2 EBV (2%) was seen only occasionally (Hu *et al.*, 1991; Chen *et al.*, 1992; Choi *et al.*, 1993). Similarly high frequencies of type 1 EBV was detected in Malaysian NPC patients (Abdel-Hamid *et al.*, 1992).

The type 2 EBV was found to be less ubiquitous than type 1 isolate (Zimber *et al.*, 1986). All type 2 isolates that have been identified occurred in Central Africa, La Reunion and New Guinea, suggested that the occurrence of type 2 was geographically restricted. There is also an increase in type 2 EBV among immunodeficient individuals (HIV patients and infected donors) than in healthy controls (Scully *et al.*, 1988).

Sixbey *et al.* (1989), provided evidence of co-infection with 2 EBV genotypes and serological findings by Scully *et al.* (1988) whereby cross-reactivity was observed between type 2 and type 1 EBNA, suggests dual infection. Yao *et al.* (1991) deduced that only a single isolate is dominant in both the oropharynx and lymphocytes of healthy carriers and no dual infection was observed.

1.8   EBV Genome

The EBV genome was first characterised by zur Hausen *et al.* (1970). It is a 172,000 base paired (bp), linear, double-stranded DNA molecule which composes of 60 percent guanine and cytosine, and codes for over 100 proteins (Figure 1.3) (Pritchett *et al.*, 1976). The EBV genome has been completely sequenced (Baer *et al.*, 1984), with promoters, open reading frames, RNAs and other structural elements mapped out (Baer *et al.*, 1984; Farrel, 1989).
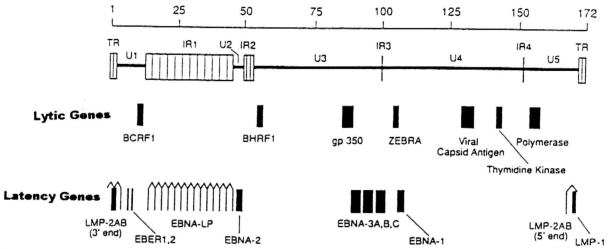
Figure 1.3: The structure of the Epstein-Barr virus genome with selected genes expressed during lytic and latency cycle (Cohen, 1997).

The genome is flanked at each end by 4-12 copies of tandem 0.5 kb long terminal repeats (TR) (Raab-Traub & Flynn, 1986), which is believed to facilitate EBV DNA circularization following host infection (Lindahl *et al.*, 1976). It is separated into short unique sequence (US) approximately 10 kb long, followed by about 40 kb internal repeat sequence (IR) and a long unique section (UL) approximately 120 kb in length. The IR sequences separate the EBV genome into five unique regions (U): U1, U2, U3, U4 and U5 DNA domains of 10, 3, 59, 40 and 30 kb respectively. The U2 region varies among the different EBV isolates. Four direct tandem internal repeat regions (IR1 to IR4) have been identified. IR1 are multiple repeats of a 3072 bp sequence. IR2 and IR3 are tandem repeats of 125 and 103 bp sequence respectively (Cheung & Kieff, 1982; Dambaugh & Kieff, 1982). The 708 bp of IR3 sequence consists of a repeat array of three nucleotide triplets: GGG, GCA and GGA (Heller *et al.*, 1982).

1.9   EBV Infection

The major target cells for EBV infection are the B-lymphocytes, in which a latent or lytic cycle of infection can occur (Rickinson *et al.*, 1979; Yao *et al.*, 1989) and stratified squamous epithelium (Sixbey *et al.*, 1984; Greenspan *et al.*, 1985). In B-lymphocytes, infection of EBV is predominantly latent, which can be triggered into the lytic cycle. EBV specific antigens can be classified into latent-phase antigens and lytic cycle antigens. Nearly 100 viral genes are expressed during the lytic cycle while only 10 are expressed in latently infected B-lymphocytes *in vitro* (Kieff, 1996). Selected genes expressed during the lytic and latent cycle are shown in Figure 1.3.

1.9.1   Latent infection

EBV enters and immortalises B-lymphocytes following interaction between the viral membrane glycoprotein gp340/220 and the B-lymphocytes receptor for the C3d complement fragment (CD21) (Jondal *et al.*, 1976). Then the viral genome circularises by covalent linkage of the terminal repeats and is maintained as multicopied episomal plasmid. Circular episomal EBV DNA is detectable in the nuclei of acutely *in vitro* infected peripheral blood lymphocytes within 24 hours (Hurley & Thorley-Lawson, 1988; Alfieri *et al.*, 1991). The replication of episomes is regulated and occurs parallel to host cell proliferation so that the genomic structure and copy numbers of episomes remain stable (Yates & Guan, 1991). In normal adults, 1 to 50 B-lymphocytes per million in circulation are infected with EBV. It remains latent over several years in the peripheral blood in resting memory B-lymphocytes in asymptomatic EBV carriers (Khan *et al.*, 1996; Babcock *et al.*, 1998).

During the latent infection stage, three families of viral specific antigens can be detected in EBV-transformed cells (Kieff, 1996). They are the Epstein-Barr virus nuclear antigens (EBNA 1, 2, 3A, 3B, 3C and LP), the latent membrane proteins (LMP 1, 2A and 2B) and EBV-encoded, small non-polyadenylated RNAs (EBER 1 and EBER 2).

The EBNA is the first group of EBV proteins after EBV infection of B-lymphocytes, detected 12-24 hours postinfection (Klein *et al.*, 1974; Einhorn & Ernberg, 1978). EBNA are also associated with chromosomes in metaphase (Reedman & Klein, 1973), with higher affinity for double stranded DNA compared to single stranded DNA (Luka *et al.*, 1977). EBNA 1 protein binds to viral DNA and maintains the EBV genome as a circular DNA episome in the B-lymphocytes (Yates *et al.*, 1984). It also blocks its own protein degradation by the ubiquitin-proteosome pathway in the cell (Levitskaya *et al.*, 1997). EBNA 2 regulates the expression of LMPs and cellular proteins for the growth and transformation of B-lymphocytes (Johannsen *et al.*, 1995). The EBNA 3 proteins (EBNA 3A, 3B and 3C) also control the expression of cellular genes (Wang *et al.*, 1990). EBNA leader protein (EBNA LP) enhances the ability of EBNA 2 to regulate LMP 1 (Wang *et al.*, 1985). EBNA LP could also be associated with EBV nuclear RNA and plays a role in EBV RNA transcription or processing (Lawrence *et al.*, 1989).

By 32 hours postinfection, all EBNAs and LMP RNAs are expressed. The LMP 1 mRNA is the most abundant EBV mRNA in latent infection, more abundant than the EBNA or LMP 2 mRNAs. By 48 hours of primary B-lymphocytes infection, all EBNA proteins and LMP are near the level that is maintained consistently through latent infection (Alfieri *et al.*, 1991). LMP 1 is a type III integral protein with six

hydrophobic transmembrane regions and distributed as patches in the cell plasma membrane. LMP 1 acts as an oncogene where its expression in transgenic mice results in B-lymphocyte lymphomas. This protein induces a signalling response in cells that mimic the active component of the B-lymphocyte surface molecule CD40. LMP 1 also up-regulates the expression of several cellular proteins that inhibit apoptosis, including bcl-2 and A20 (Kulwichit *et al.*, 1998; Uchida *et al.*, 1999). *In vitro* and *in vivo*, LMP 1 binds to several tumour necrosis factor receptor and associated factors. This results in activation of nuclear factor-(kappa)B transcription factor (NF-κB), activation of c-jun, up-regulation of cellular adhesion molecules, cytokine production and B-lymphocytes proliferation (Mosialos *et al.*, 1995; Liebowitz, 1998). LMP 2B protein is identical to LMP 2A but lacks the hydrophilic N terminus (Laux *et al.*, 1988). The LMP 2B may act to modulate the aggregating effects of LMP 2A. The LMP 2 proteins prevent reactivation of the EBV lytic cycle from latently infected cells by blocking tyrosine kinase phosphorylation (Miller *et al.*, 1995).

With an estimation of 10^5- 10^7 copies per cell, the EBERs are the most abundant EBV RNAs in latently infected cells. Most of the EBERs localise at the cell nucleus, complexes with cellular proteins (Howe & Steiz, 1986). EBERs expression lags by approximately 24 hours after EBNA and LMP gene expression and the onset of cell synthesis. It only reaches substantial levels 70 hours postinfection (Alfieri *et al.*, 1991). EBER do not encode proteins, but may be important for oncogenesis and resistance to apoptosis (Komano *et al.*, 1999).

1.9.2 Lytic infection

The EBV infected cells enter the lytic cycle when the latent stage is disrupted. The BamH1 Z EBV replication activator (ZEBRA) protein triggers the switch from latent to lytic infection. ZEBRA protein mediates the genetic switch by activating two EBV regulatory gene promoters (BMLF1 and BMRF1) that encode the transactivating factors for the production of new virus particles (Countryman & Miller, 1985; Kenny *et al.*, 1989; Taylor *et al.*, 1989). The activation results in cell death and released of viral progeny (Facer & Playfair, 1989). The latency of EBV in lymphoblastoid cell lines (LCLs) is however under tight control. Only a minor fraction (1 in 10^2-10^6 cells) in LCLs cultures switch from latent to lytic infection (Klein & Dombos, 1973; Sugden *et al.*, 1979). Lytic infection can be initiated *in vitro* by utilising phorbol esters or calcium ionophore or cross-linking of surface Igs (zur Hausen *et al.*, 1978; Luka *et al.*, 1979). Another approach to induce viral replication is by superinfection of Raji cells with a latent antigen-defective EBV from P3HR-1 cell line (Biggin *et al.*, 1987).

The lytic infection is characterised by extensive transcription of viral genome with subsequent antigen expression. Because virus gene expression in lytic infection is at a much higher level than in latent infection, lytic cycle proteins are predominantly over latent proteins. The antigens include membrane antigen (MA) (Klein *et al.*, 1966), the viral capsid antigen (VCA) (Pearson *et al.*, 1971) and the early intracellular antigen (EA) (Henle *et al.*, 1970b).

EBV MA was first demonstrated in Burkitt's Lymphoma (BL) biopsy cells by membrane immunofluorescence (Klein *et al.*, 1966). The MA was later detected in the plasma membranes of viable cells infected with the virus supporting the replication of virus particle (Klein *et al.*, 1966; Pearson, 1980). It is synthesised as both early and

late viral replicate cycle antigens (Ernberg *et al.*, 1974). MA is also expressed in the envelopes of mature virus particles (Silvestre *et al.*, 1971) and can induce EBV-neutralising antibodies (Pearson *et al.*, 1978). The function of MA is attributed to the viral envelope include the binding and adsorption of the virion to receptors on the target B-lymphocyte. This protein also mediates the fusion of the virus envelope to the membrane that facilitates the release of viral DNA into the cells (Miller & Hutt-Fletcher, 1988).

1.10 EBV Antigens

1.10.1 Viral capsid antigen (VCA)

The viral capsid antigen (VCA) was the first antigen detected using immunofluorescence assay (IFA) test in virus producing cell lines such as P3HR-1 and EB-3. A small percentage of cells in the culture were brilliantly stained when reacted against various human sera (Henle & Henle, 1966).

The VCA complex expression depends on viral DNA synthesis as inhibition of DNA synthesis inhibits VCA synthesis (Summers & Klein, 1976). VCA synthesis is a late function of the viral genome in infected culture cells and appeared late in the virus replication cycle (Henle *et al.*, 1970a; Pearson *et al.*, 1971). VCA composed of polypeptides associated with the capsid and envelope of the complete virus particle (Pearson *et al.*, 1971). The major polypeptides have molecular weights of 125 kD, 152 kD and 160 kD. Monoclonal antibodies against the 125 kD and 160 kD polypeptides have been produced and characterised (Takada *et al.*, 1983; Kishishita *et al.*, 1984; Vroman *et al.*, 1985; Uen *et al.*, 1988).

The 125 kD polypeptide is encoded by the BamH1 A fragment and appeared late in the virus replication cycle. This protein is present in the nucleus and cytoplasm of virus producing cells and also in extracts of purified nucleocapsids (Pearson & Luka, 1986). The 125 kD protein is glycosylated and the carbohydrate moiety was shown to be complex in nature and rich in mannose (Kishishita *et al.*, 1984). In contrast, the 160 kD protein encoded by BamH1 C fragment is non-glycosylated and located mainly in the nuclei of infected cells (Vroman *et al.*, 1985). Dolyniuk *et al.* (1976) demonstrated that the 160 kD protein was the major protein in solubilised EBV nucleocapsids.

All sera from EBV infected individuals reacted with the purified 125 kD glycoprotein in enzyme-linked immunosorbent assay (ELISA) (Luka *et al.*, 1984). However, not all sera from EBV infected individuals reacted with the 160 kD protein (Vroman *et al.*, 1985). Therefore, the 125 kD protein appears to be the major immunogen following primary infection with EBV even though the 160 kD protein is the major protein composing the viral capsid.

1.10.2 Early antigen (EA)

The synthesis of early antigen (EA) denotes the beginning of EBV lytic cycle. This viral protein is postulated to be involved in the initiation of viral lytic cycle and the shutdown of host-cell functions (Thorley-Lawson *et al.*, 1982). EA appeared approximately 6-8 hours after infection and about 4 hours before VCA. Polypeptides with molecular weights from 30 kD to 165 kD have been classified as early proteins (Pearson, 1980). The proteins associated with the EA complex are presumably used in early stages in the replication of viral DNA. These includes DNA polymerase (Datta

et al., 1980; Allaudeen & Rani, 1982), DNase (Cheng *et al.*, 1980), thymidine kinase (de Turenne-Tessien *et al.*, 1989) and ribonucleotide reductase (Ginsburg, 1990).

The synthesis of EA is neither dependent or inhibited by viral DNA synthesis (Henle *et al.*, 1973). The EA consists of two components designated as diffused (EA-D) and restricted (EA-R) based on sensitivity to methanol fixing and staining patterns. The EA-D is resistant to ethanol or methanol fix and found in both the nucleus and cytoplasm. The other component, EA-R is localised in a large mass in the cytoplasm of acetone fixed cells adjacent to the nucleus. The EA-R component is destroyed by ethanol or methanol (Henle *et al.*, 1971).

The major polypeptides identified have molecular weights from 47 kD – 60 kD, 85 kD and 140 kD. Monoclonal antibodies against the 47 kD – 60 kD complex and 85 kD polypeptide have been produced (Pearson *et al.*, 1983a; Epstein, 1984). The 47 kD – 60 kD polypeptide encoded by the BamH1 M fragment is a DNA-binding phosphoprotein (Pearson et al., 1983a), while BamH1 O right fragment 2 codes for the 85 kD polypeptide, a non DNA-binding polypeptide (Goldschmidts, 1987). By immunofluorescence staining, the antibody against the 47 kD – 60 kD reacted mainly with the nucleus of infected cells classifying it as the EA-D component. The 85 kD protein reacted largely with granular and fibrous or filamentous structures in the cytoplasm of infected cells classified as the R component.

The presence of IgG/EA reflects an active infection although this antigen is not expressed in EBV-carrying tumour cells (Young *et al.*, 1988).

1.10.3 BHRF1 protein

The EBV BHRF1 protein is an immediate early protein and the gene is found to be present in all natural EBV isolates examined. Its sequence is highly conserved suggesting some crucial role for this protein in the virus life cycle (Heller *et al.*, 1981). The BHRF1 lytic gene (Pearson *et al.*, 1987) encodes a 17 kD component of the EBV-restricted early antigen complex (EA-R). The BHRF1 protein has significant co-linear homology alignment of amino acid sequences (38%) with bcl-2, a cellular proto-oncogene. The cellular distributions of both proteins are located at the periphery of mitochondria (Cleary *et al.*, 1986; Hickish *et al.*, 1994). The BHRF1 protein was shown to be a functional analogue of the bcl-2 protein in its ability to prevent programmed death of human B-lymphocytes (Henderson *et al.*, 1993; Dawson *et al.*, 1995). The gene products of BHRF1 and bcl-2 are functionally similar to each other.

The BHRF1 protein protects cells from apoptosis induced by several stimuli such as tumour necrosis factor, anti-Fas antibody, monocyte killing and the c-*myc* proto-oncogene (Fanidi *et al.*, 1998). Nevertheless, the BHRF1 antiapoptotic effects are often cell type dependent (Foghsgaard & Jaattela, 1997). Like its cellular homologue bcl-2, BHRF1 blocks apoptosis in lymphocytes and epithelial cells *in vitro* but unlike bcl-2, BHRF1 enhances epithelial cell survival under low serum conditions during *in vitro* culture (Dawson *et al.*, 1998) or by addition of calcium ionophore or various DNA-damaging agents (Tarodi *et al.*, 1994).

BHRF1 protein has been postulated to be closely associated with the development of NPC through prevention of apoptosis of EBV-infected cells during the early stages of cancer development. Therefore, there may be increased expression of BHRF1 protein in NPC patients (Horner *et al.*, 1995).

1.11   EBV Serology in NPC

The immunological response of EBV infected patients to different virus specific antigenic markers can be characteristic of the type of EBV-associated disease and is therefore of diagnostic and prognostic significance (Table 1.2).

Table 1.2: EBV-specific antigens and immunological response in patients with EBV-associated disease (Tam & Murray, 1990; Tam, 1991).

Antigen	Latent infection	Lytic infection	Immune response	Disease association
EBNA	+	+	IgG	Past infection
EA-D	-	+	IgG	IM, NPC
			IgA	NPC
EA-R	-	+	IgG	BL
VCA	-	+	IgG	Recent or past infection
			IgM	Primary infection, IM
			IgA	NPC
MA	-	+	IgG	Past infection
			IgA	NPC[a]

NPC – nasopharyngeal carcinoma

IM – infectious mononucleosis

BL – Burkitt's lymphoma

[a] - has been suggested, but not confirmed by independent reports

More than 90% of NPC patients have elevated antibodies to EBV determinant antigens. The presence of high antibody titres to EBV antigens in NPC patients is a useful aid for diagnosis of NPC especially when tumours are small and confined to the submucosa (Neel *et al.*, 1985; Chew, 1990).

The titres of EBV antigens, particularly IgA/VCA, were found to be elevated in NPC compared to other cancers, including the head and neck cancers (Henle *et al.*, 1970a). IgA/VCA has been reported to correspond to the tumour burden and elevated even before clinical appearance of NPC. If a cancer is detected early, the high costs of multi-modality treatment required for advanced disease and managing complications can be reduced. Treatment for early cancer is more effective and is often much

simpler with lower doses of radiotherapy. In a study from China, the antibodies to the EBV viral capsid antigen (VCA) and early antigen (EA) were found to be present for at least a year prior to the development of clinically detectable disease (Zeng *et al.*, 1983b). This finding has not been substantiated by any study out of China, including Malaysia (unpublished data from the NPC Lab in University Malaya, Kuala Lumpur).

The IgA/VCA is however a diagnostic marker of NPC and is used as an aid to the clinical diagnosis of NPC, particularly in cases where the lesion is obscure. In a study by Sam *et al.* (1989), 8 patients with clinical symptoms of NPC were biopsied but were found histopathologically normal. All 8 had had elevated IgA/VCA titres. Repeat biopsies were carried out and NPC cells were found in all 8 cases. This indicated that false negative diagnosis can be made due to unrepresentative biopsy samples or the histological features of the biopsied samples were obscured by traumatic distortions or necrosis (Shanmugaratnam, 1984).

Numerous studies have found IgA/VCA to be a useful diagnostic marker for NPC. The sensitivity and specificity values are the most important parameters in choosing the best diagnostic tests. Reports of the sensitivity and specificity values are summarised as in Table 1.3.

Table 1.3: Sensitivity and specificity of IgA/VCA in the diagnosis of NPC

Sensitivity (%)	Specificity (%)	Reference
93.0	95.0	Henle & Henle, 1976
70.0	98.3	Hadar *et al.*, 1986
81.4	95.8	Sam *et al.*, 1989
84.6	84.5	Puthavathana *et al.*, 1993
90.6	73.3	Chan *et al.*, 1998
100.0	91.7	Shimakage *et al.*, 2000

Other EBV markers, such as IgG and IgA/EA, are also used as complementary markers of NPC. IgA/VCA and IgA/EA are used as routine diagnosis aids for NPC at the World Health Organisation (WHO) Immunology Collaborating Centre, Singapore. The IgA/EA is a more specific (95.7%) but less sensitive (79.7%) marker compared to IgA/VCA with sensitivity of 90.6% and specificity of 73.3% (Chan *et al.*, 1998). Puthavathana *et al.* (1993) also reported IgA/EA to be more specific (87.2%) but less sensitive (79.1%) compared to IgA/VCA. Moreover, IgA/EA was found only in individuals positive for IgA/VCA (Ho *et al.*, 1981; Zeng *et al.*, 1982). However, using the sensitive immunoautoradiographic technique, sensitivity of IgA/EA was increased to a 4% of false negative (Zeng *et al.*, 1983a). The usage of IgG/EA as an adjunct to IgA/VCA has been reported to be useful for increasing sensitivity of NPC diagnosis (Pearson *et al.*, 1983c; Sam *et al.*, 1989). Specificity may be increased by having more than one diagnostic marker as the percentage of false positive for one antibody is expectedly higher than for two or more antibodies considered simultaneously.

Pearson *et al.* (1983b) established the clinical value of EBV serology for the diagnosis of different histopathological types of North American NPC including primary occult tumours. They found patients with WHO I type generally had a low EBV serological profile and were similar to those found in upper aerodigestive tract squamous cell carcinomas patients and other control populations. Nucleic acid studies demonstrated that WHO I type is rarely associated with EBV (Raab-Traub *et al.*, 1987). However, a large majority of patients with WHO II and WHO III carcinomas have elevated IgG/EA and IgA/VCA (Krueger *et al.*, 1981; Pearson *et al.*, 1983b).

Eighty three percent of sera with WHO II and WHO III carcinomas were positive for IgA/VCA in contrast to only 19% of sera from WHO I patients. Eighty

six percent were positive for IgG/EA as opposed to 38% of the sera from WHO I patients (Pearson *et al.*, 1983b). In contrast, Sam *et al.* (1989) reported IgA/VCA and IgG/EA to be similarly elevated in majority of all three histopathological types of NPC. Only 9 WHO I samples were studied as WHO I is rare. The data may be biased due to the insufficient number of samples. The authors also suggested that the lack of standardise histopathological classification of NPC may caused the pathologist to wrongly classified the samples.

EBV serology assays can be carried out using the major EBV antigen complexes or an individual specific EBV polypeptide. The various antigen complexes are broadly categorised. Genetic engineering technology has enabled the production of polypeptides from cloned EBV genes. Synthetic peptide technology has also made possible the availability of defined EBV associated antigen. These polypeptides serve as target antigens in the enzyme-linked immunosorbent assay (ELISA). Polypeptides of EBV that showed high potential as diagnostic serological markers for NPC are summarised as in Table 1.4.

Table 1.4 Potential individual polypeptides of the antigen complexes of EBV for the diagnosis of NPC

Polypeptides	Immune response	Reference
Viral capsid antigen (VCA)		
160 kD protein, p160	IgG	Vroman *et al.*, 1985
125 kD protein, gp125	IgA	Uen *et al.*, 1988
minor polypeptide, p18	IgG	Uen *et al.*, 1988
Early antigen-diffused (EA-D) complex		
polypeptide, pp58	IgG	Chen *et al.*, 1991
Early antigen-restricted (EA-R) Complex		
ribonucleotide reductase,	IgG	Fones-Tan *et al.*, 1994
large subunit, 85 kD	IgA & IgG	Gan *et al.*, 1999
DNase, 52 kD	IgA	Chen *et al.*, 1993
thymidine kinase, 67 kD	IgA	Littler *et al.*, 1991
BHRF1 antigen, 17 kD	IgG & IgA	Liu *et al.*, 1998
Early antigen (EA) unidentified		
ZEBRA antigen	IgG	Mathew *et al.*, 1994
	IgG	Dardaari *et al.*, 2000
Membrane antigen (MA) Complex		
gp 250/200	IgA	Durda *et al.*, 1993
gp 350/220	IgA	Xu *et al.*, 1998
Latent Infection Protein		
EBNA1 (p107)	IgA	Cheng *et al.*, 1993

1.12 Epitope Mapping Using Synthetic Peptides

An epitope or antigenic determinant is the structural element of recognition in antigen-antibody complex, is highly specific for macromolecular recognition. Epitopes can be either linear (continuous), made up of a linear formation of amino acid residues, or assembled (discontinuous), made up of residues from different loops of peptide brought together by the folding of the protein (Atassi, 1984; Geysen, 1985).

Epitope mapping of B-cells and T-cells are for understanding the nature of antigenic recognition of the immune response and an essential part of producing protective peptide vaccines. B-cells epitope mapping in particular is an essential element in understanding the pathology of major diseases. For detection of serum antibodies to pathogens, it is of clinical relevance to locate and define epitopes, as the use of epitope-peptides increases the specificity of diagnostic immunoassays (Geysen et al., 1987b). A fully intact B-cell epitope is not necessarily required for the interaction between the antibody and epitope. Parts of the epitope as linear peptides may bind the antibody, although at lower affinity. Therefore, although linear peptides will rarely represent full epitopes, they can be used to map B-cell epitopes (Meloen et al., 1988).

Epitope mapping can be carried out by prediction from amino acid sequence of a protein, inferred from the location of amino acid mutations which disrupt antibody binding, assessed by amino acid sequencing of antibody binding clones of genomic expression libraries or assessed using peptides. Epitope mapping with peptides will remain a simple and important approach (Bosshard, 1995; Meloen et al., 1995). However, epitope mapping with short synthetic peptides homologous with the sequence of the protein antigen is generally limited to linear epitopes. Strategies for

reconstructing assembled epitopes using linear peptides are not successful in many cases (Rodda & Tribbick, 1996).

Synthetic peptides are potent tools for a broad spectrum of applications in biological research. The peptides can be chemically synthesised on solid phase or solution phase. Peptides on solid phase are more favourable. Solid phase peptide synthesis, pioneered by Merrifield (1963), involves the successive addition of amino acids to create a linear peptide chain. Merrifield used a polar solid support (beaded polystyrene), a protecting group strategy based on the mildly acid labile t-butyloxy-carbonyl group (Boc) for α-amino acid protection, benzyl-based side chain protection, a benzyl ester linkage to the resin and *in situ* activation with dicyclohexylcarbodiimide. At the end of an assembly, treatment with a strong acid (hydrogen fluoride) is required to remove the peptide from the solid support and effect side-chain deprotection.

Atherton *et al.* (1978) adopted a milder and more flexible method, based on the base labile fluorenylmethyloxycarbonyl (F-moc) group for α-amino acid protection. The acid labile t-butyl group is used for the side chain group. This method employs N,N-dimethylformamide (DMF) for washing and 20% piperidine in DMF to remove F-moc group. At the end of the assembly a single trifluoroacetic acid (TFA) treatment is used to cleave the peptide from the support and remove the side-chain protecting group.

In a synthesis, the C-terminus of the growing peptide is covalently bound to a solid support. The amino acids used in a synthesis are protected to prevent unwanted reactions at the α-amino and side-chain functions. Three chemical reactions are

repeated for each amino acid that is added to the peptide chain: deprotection, activation and coupling (Figure 1.4).
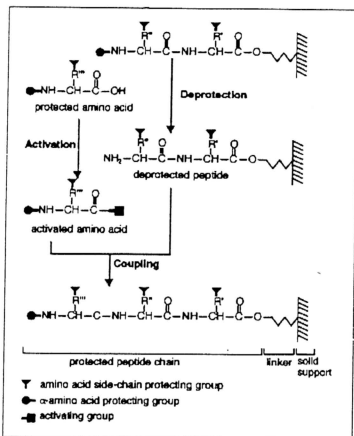

Figure 1.4: Solid phase peptide synthesis: a cyclical, three-step process.

Deprotection: During the deprotection, the protecting group is removed to able accessibility of the α-amino on the end of the peptide chain.

Activation: Activation changes the next amino acid to be added to an active ester. For α-F-moc protected amino acid, the protected amino acids are activated *in situ* by addition of the additive, 1-hydroxy-benotriazole (HOBt) and the activator diisopropylcarbodiimide (DIC).

Coupling: Coupling is the peptide forming reaction, which the active ester forms an amide bond with the deprotected α-amino group on the end of the peptide chain to form new peptide bond.

Then, a new cycle of synthesis begins with the next deprotection. After the last amino acid is coupled, the N-terminus of the peptide is acetylated to remove the charged associated with a free terminal amino acid group and all the side chains of the amino acids are deprotected.

1.12.1 Multipin peptide synthesis

Geysen *et al.* (1984; 1987a) reported a novel approach of solid phase peptide synthesis which, made it possible to map systematically the entire sequence of a protein with overlapping peptides: the PEPSCAN method. This method produces nanomole amounts of peptides covalently attached to polyethylene pins that fit into the wells of a standard 96-wells microtiter plate. All the synthesis steps are performed on the pins immersed in the wells and the peptide is not cleaved from the support after completion of synthesis. The polymer-bound peptides are used directly to incubate with the antibody, washed, incubated with an enzyme-coupled secondary antibody and processed with a substrate.

Peptides on solid phase are very convenient for testing in a simple binding assay (Multipin ELISA) as testing is done on the same surface used for synthesis. However, the use of peptides in this way can lead to difficulties based on uncertainty as how well each peptide is captured or whether the peptide is present in a form that can interact with a macromolecule (Geerlings *et al.*, 1988). Thus, the possibilities of false negative or false positive results are significant disadvantage of passive coating

methods and methods in quality and quantity of each peptide is not assessed (Rodda *et al.*, 1986; Trifilieff *et al.*, 1991)

Numerous epitopes have been mapped using Multipin synthetic peptides such as the viral hemorrhagic septicemia virus (VHSV) Glycoprotein G (Estepa & Coll, 1996), epitopes of the X protein of Hepatitis B virus (Kumar *et al.*, 1998), binding sites on enzymes (De Silva *et al.*, 1999), thymosin beta 10 protein (Vassiliadou *et al.*, 1999), human brain acetylcholinesterase (Zhang *et al.*, 2000) and human zona pellucina glycoprotein-B (Govind *et al.*, 2000). For EBV proteins, the linear epitopes of the replication-activator protein of EBV (Cheng *et al.*, 1995) and epitope clusters of the EBV major capsid protein (Middeldorp & Meloen, 1988) has been mapped out using the Multipin method.

1.13   Objectives of This Study

There are two objectives in this study:

1) To determine the sensitivity and specificity of IgA and IgG to Epstein-Barr virus viral capsid antigen (EBV-VCA) and IgA and IgG to Epstein-Barr virus early antigen (EBV-EA) in sera of NPC patients from Sarawak

2) To define antigenic epitopes of the BHRF1 protein in these NPC patients through peptide synthesis and enzyme-linked immunosorbent assay (ELISA).