# CHAPTER 4

# DISCUSSION

## 4.1 Incidence of NPC in Sarawak

Substantial work on incidence and EBV serology of NPC in Malaysia has been done in West Peninsular Malaysia. Not much has been reported of East Malaysia, which is situated in the Island of Borneo. In the study on Malaysian NPC by Norhanom (1989), 20 NPC serum samples were obtained from Sarawak and all were collectively referred to as Ibans.

In this study, the sera were random samples collected from newly histologically confirmed NPC patients who enrolled at the Radiotherapy Unit (RTU), Kuching General Hospital, Sarawak. In Sarawak, NPC was more common in males, the male/female ratio was found to be 3.4 : 1. In Peninsular Malaysia, male/female ratio of 2.8 : 1 was reported by Prasad & Rampal (1992) . A ratio of 3.5 : 1 was reported in NPC patients in Israel (Hadar *et al.*, 1986). In Singapore and Hong Kong, male/female ratio of 2.4 :1 and 2.6 : 1 was reported (Parkin *et al.*, 1997).

Incidence of NPC Sarawak is low among those below 20 years of age. The incidence begins to rise in the second decade of life. The peak morbidity occurred in the fourth decade (40-49 years age group). Mean age of NPC patients was 47.9 years. Prasad & Rampal (1992) reported the same peak age group in Peninsular Malaysia. In Sarawak, a wide diversity of ethnic groups is observed. The Ibans, which make up the highest population in Sarawak (29.6%) constituted the highest number of NPC patients (42.1%) followed by the Chinese (22.5%), which make up of 27.4% of the

population. Relative to the population size, the Ibans had higher incidence of NPC cases compared to the other ethnic groups.

The Ibans originated from the Kapuas basin and the coastal regions of West Kalimantan. They are the largest community in Sarawak and located mainly in the central area of the state. The Ibanic people are quite different in culture, language and social order from the other indigenous groups (King, 1993). The Chinese population of both Peninsular and East Malaysia is largely derived from South China. In Sarawak, most of the Chinese came from Kwantung and Fukien provinces of Southern China and brought their customs, religions and languages. The Chinese are ranked as the second largest community and can be located in all the nine divisions in the state, centralising in the urban areas (Chang, 1993).

The Southern Chinese are known to be the high-risk group for NPC. In a multi-racial society like Malaysia, the cultural interaction and intermingling may influence some changes in customs, observances, languages and even religions. However, in general each ethnic group still retains some practices peculiar to that group and intermarriages are rare. Therefore, genetic predisposition and cultural factors can contribute to the development of a cancer. The high incidences among the Ibans merits further studies to identify the factors involved in the development of NPC.

## 4.2 Detection of Anti-Epstein-Barr Virus Antibodies

The Epstein-Barr virus (EBV) is closely associated with nasopharyngeal carcinoma (NPC), since all NPC biopsies have been found to contain EBV nucleic acids and the majority of NPC patients have elevated antibody titres to EBV proteins. EBV infects 2 human cells: B-lymphocytes and epithelium cells. EBV infection in B-lymphocytes can be latent or lytic. The role(s) of EBV in NPC remains unclear but antibodies to EBV markers particularly the lytic proteins - the viral capsid antigen (VCA) and early antigen (EA) have been shown to be useful identifying markers of NPC, which in the majority of cases are difficult to diagnose. The IFA method is the method of choice for the detection of these antibodies.

The findings of the present study are in accordance with many previous reports (Henle & Henle, 1976; Ho *et al.*, 1976; Pearson *et al.*, 1983b; Sam *et al.*, 1989; Puthavathana *et al.*, 1993; Chan *et al.*, 1998). NPC patients have elevated antibodies to EBV lytic cycle proteins, VCA and EA. The GMTs of the 4 anti-EBV antibody titres for NPC patients were observed to be 34.8, 412.4, 14.9 and 76.0 for IgA/VCA, IgG/VCA, IgA/EA and IgG/EA, compared to 2.3, 9.6, 2.0 and 3.1 for controls (Table 3.8).

### 4.2.1 Anti-EBV antibodies in NPC patients with respect to sex, ethnic groups and age

IgA/VCA, IgG/VCA, IgA/EA and IgG/EA titres showed no significant difference with respect to the sex or age of patients. The patients were grouped into 5 ethnic groups, one of the group was a pooled group of indigenous minorities. These groups could be genetically and geographically different, but due to the small number

of samples (17 cases), they were grouped together. A marked difference in NPC prevalence with reference to ethnic group was observed but no ethnic variation on EBV antibodies was seen among the various groups.

### 4.2.2 Sensitivity and specificity of anti-EBV antibody assays

EBV infection is ubiquitous and EBV seroconverted individuals carry the EBV genome in a latent state (Khan *et al.*, 1996; Babcock *et al.*, 1998). EBV seroconversion is 100% by the time Malaysian children are 9 years old (Chua, 1999). EBV genomes are activated regularly and EBV particles released into circulation would induce an immune response against the viral antigens in healthy seroconverted individuals (Rickinson *et al.*, 1985). Their antibody levels are normally detectable at low titres. In some individuals however, slightly elevated titres are sometimes detected. In healthy family members of NPC patients, there is a tendency to show antibodies level especially of IgA/VCA that are higher compared to the general population (Ho *et al.*, 1978).

The sensitivity and specificity values are the most important parameters in the determination of accuracy and reliability of the test. Sensitivity is how good a test detects a disease without missing some diseased individuals by falsely classifying them as healthy. Specificity indicates how well a test is at detecting the diseased individuals without falsely labeling some healthy persons as having the disease. The specificity of a test reflects its ability to detect true negatives. The cut-off titre for the anti-EBV antibody markers was determined by considering its sensitivity and specificity.

In this study, it was found that the best separation of NPC patients from controls was obtained using titres of ≥10 for IgA/VCA, ≥160 for IgG/VCA, ≥5 for IgA/EA and ≥40 for IgG/EA. The GMTs of the markers for NPC patients above the cut-off titres are 55.6, 576.5, 29.0 and 166.3 for IgA/VCA, IgG/VCA, IgA/EA and IgG/EA. Different reports have different cut-off titre of EBV serological titres as measured by immunofluorescence assay (IFA) for the diagnosis of NPC (Table 4.1).

Table 4.1: Various cut-off titre of EBV measured by IFA

| Marker | Cut-off titre | Reference |
|---|---|---|
| IgA/VCA | ≥ 10 | Ho *et al.*, 1978 |
| | | Pearson *et al.*, 1983b |
| | | Neel *et al.*, 1985 |
| | | Sam *et al.*, 1989 |
| | | Puthavathana *et al.*, 1993 |
| | | Chan *et al.*, 1998 |
| | | Present study |
| | ≥ 40 | Lynn *et al.*, 1985 |
| IgG/VCA | ≥ 60 | Pearson *et al.*, 1983b |
| | ≥ 160 | Henle & Henle, 1976 |
| | | Ho *et al.*, 1978 |
| | | Present study |
| | ≥ 640 | Lynn *et al.*, 1985 |
| IgA/EA | ≥ 5 | Present study |
| | ≥ 10 | Zeng *et al.*, 1982 |
| | | Chan *et al.*, 1998 |
| IgG/EA | ≥ 10 | Pearson *et al.*, 1983b |
| | | Neel *et al.*, 1985 |
| | | Sam *et al.*, 1989 |
| | ≥ 40 | Present study |

Using these titres (≥10 for IgA/VCA, ≥160 for IgG/VCA, ≥5 for IgA/EA and ≥40 for IgG/EA), IgG/VCA was the most sensitive (89.0%) in distinguishing NPC patients. As the titres of the patients are substantially higher than those of healthy controls, high titres of IgG/VCA have been considered as potential screening marker (Hadar *et al.*, 1986). However, IgG/VCA antibodies are known to be present in other EBV infections such as infectious mononucleosis, asymptomatic EBV infection, reactivation of latent EBV infection and Burkitt's lymphoma (Okano *et al.*, 1988). IgG/VCA was shown to be elevated (titre >160) in 25% of control group including healthy individuals and patients with other head and neck diseases (Neel *et al.*, 1980). In this study, only NPC patients and non-NPC controls were compared. Other EBV related diseases were not taken into consideration, which likely raised the specificity of IgG/VCA as a marker of NPC.

The most specific antibody was IgA/EA (100.0%) since no controls had any detectable IgA/EA. The sensitivity and specificity of IgA/VCA was 83.6% and 97.3% respectively. IgA/VCA is thus more sensitive but less specific than IgA/EA. Similar findings had been reported by Zeng *et al.* (1982) and Chan *et al.* (1998). IgA/VCA in particular has been shown to be highly specific for NPC. IgA/VCA is rarely observed in healthy individuals. Generally not more than 15% of 591 controls including healthy individuals and patients with other head and neck diseases have elevated level for IgA/VCA (Pearson *et al.*, 1983b). IgA/EA and IgG/EA were slightly more specific as markers compared to IgG/VCA and IgA/VCA but less sensitive.

### 4.2.3 Evaluation of anti-EBV antibody profile

The IgG/VCA response provided the best combination between sensitivity and specificity. It was therefore used as a reference for comparison with the other 3 markers (IgA/VCA, IgA/EA and IgG/EA). A significant positive correlation between IgG/VCA and the other 3 anti-EBV markers was found (Figure 3.16, 3.17 and 3.18). In a majority of cases, if a patient has high IgG/VCA titre, the other anti-EBV titres would also be elevated. The correlation coefficient (r) measured the strength of association between the EBV serological markers.

Between IgG/VCA and IgA/VCA, the correlation coefficient was 0.61. This means that 37.1% of patients with positive IgA/VCA titre is positively correlated with positive IgG/VCA titre. The correlation coefficient between IgG/VCA and IgA/EA was 0.59 indicating 34.8% of the patients' IgA/EA and IgG/VCA titre increase together. For IgG/VCA and IgG/EA, correlation coefficient was 0.76, meaning 57.8% of IgG/EA titre is positively correlated with IgG/VCA titre.

Here, a series of test were conducted (IgA/VCA, IgG/VCA, IgA/EA and IgG/EA) to determine if a battery of test will significantly increase the sensitivity and specificity over a single antibody diagnosis marker test. All 4 antibodies at appropriate elevated titres, were strongly associated with NPC and can therefore be used as markers for the diagnosis of NPC. One hundred and twelve (68.3%) of the NPC patients possessed all 4 antibodies, while 14 (8.5%) patients were negative to all antibody markers. Eighteen (11.0%) of the patients had positive titre for 3 markers, 10 (6.0%) patients were positive for 2 markers and 10 (6.0%) patients only had positive titre for 1 marker (Table 3.14).

In 27 (16.5%) of the 164 NPC patients, IgA/VCA was negative even though clinical and histopathological evidence were positive. This phenomenon of negative IgA/VCA (titre <10 as measured by immunofluorescence assay) in NPC patients was also reported by other researchers (Table 4.2).

Table 4.2: Percentage of negative IgA/VCA titre in NPC confirmed patients

| Patients | Percentage (%) | Reference |
|---|---|---|
| American | 31.0 | Pearson *et al.*, 1983b |
| Hong Kong | 6.8 | Ho *et al.*, 1976 |
| West Malaysian | 19.0 | Sam *et al.*, 1989 |
| Singaporean | 9.4 | Chan *et al.*, 1998 |
| Thai | 15.4 | Puthavathana *et al.*, 1993 |
| Sarawak (East Malaysian) | 16.5 | Present study |

The absence of IgA/VCA antibody in these patients may be due to insufficient antigenic stimulation. It has been suggested by Ho *et al.* (1976) that the IgA antibody response to VCA is under dual control, due to stimulation with antigens originating from the NPC cells and to an autosomal recessive gene prevalent among NPC patients and their family members. The autosomal recessive gene may determine and increase the capacity to mount the IgA/VCA antibody response. This causes the elevation of serum IgA/VCA concentration in NPC patients as compared to healthy individuals. Even though the IgA antibody response is increased, it is still limited, relative to that of the IgG immune system. The serum IgG concentration is about four times more than that of IgA, probably implying that the IgA antibody response is more easily saturated by the antigenic load (Ng *et al.*, 1978).

If only IgA/VCA was used, 137 out of 164 (83.6%) NPC cases were detected. By using together with another complementary marker, the sensitivity for cases positive for both or either marker was increased (Table 3.14). Though highly specific,

IgA/EA is not a good complementary marker to be used together with IgA/VCA. The 75.0% patients positive for IgA/EA were also the same patients positive for IgA/VCA. Therefore, the sensitivity and specificity of combination of IgA/VCA and IgA/EA remained the same as the using IgA/VCA alone (Table 3.12 and 3.15). In some cases, patients had elevated IgA/VCA but negative IgA/EA. Example; one serum (CCRID 3018) with IgA/VCA titre of 40 but IgA/EA titre of <5. This is a well-documented phenomenon as anti-EA antibodies are transient. Henle & Henle (1976) reported that IgA/EA was found in 70% of NPC patients with IgA/VCA. Additionally, IgA/EA was found only in individuals positive for IgA/VCA (Zeng *et al.*, 1982). Sigel *et al.* (1994) found that IgA/EA are present in other diseases like non-Hodgkin lymphoma and chronic lymphatic leukaemia but in lower titre (GMT=45.8) compared to NPC patients (GMT=145.0).

By using other combination of 2 antibodies, the sensitivity for cases positive for both or either marker was increased (Table 3.15). IgG/EA have been used concomitantly with IgA/VCA for increasing sensitivity (Lanier *et al.*, 1981, Sam *et al.*, 1989). When IgA/VCA and IgG/EA were used, the sensitivity percentage rose to 87.2% for cases positive for both or either marker but the specificity decreased to 96.6% for cases negative for both markers. A higher sensitivity percentage 90.9%, (149 out of 164) of cases was detected positive for either IgA/VCA or IgG/VCA or both markers. However, specificity decreased to 95.2% when both IgA/VCA and IgG/VCA were used. The 2 assays test of IgA/VCA and IgG/VCA should be done together as they are clearly complementary.

## 4.3 Epitope Analysis of EBV BHRF1 Protein

The EBV BHRF1 protein has been suggested to prevent apoptosis of EBV-infected cells during the early stages of NPC carcinogenesis (Horner *et al.*, 1995). In this study, an attempt was made to map the antigenic epitopes of the 17 kD, 191 amino acid residues BHRF1 protein by the Multipin peptide synthesis approach. The original approach of Multipin peptide synthesis (Geysen *et al.*, 1984; 1987a) has since been refined. The Multipin peptide synthesis kit from Chiron Mimotopes Peptides System was utilised. Meloen *et al.* (1995) reported peptides of 8–9 amino acids long were more useful for epitope mapping than the original assumption that all linear epitopes were 6–7 amino acids long. Thus, a set of 46 peptides with 10 amino acid residues overlapping by 4 residues were chosen here.

The Multipin BHRF1 peptides function as antigens in the antigen-IgA antibody binding assay. Few of the 46 synthetic peptides used in this study showed strong distinct binding ability to IgA antibodies in NPC sera. However, the specific antibody binding profile (Figure 3.23) indicated that peptides 6, 17, 26, 27 and 29 were more reactive with NPC sera. Peptide 17 in particular had the highest specific antibody binding in 4 out of 5 NPC sera (Table 3.16). This peptide with the amino acid sequence of FTETWNRFIT merits further investigation.

The high background binding observed make deduction of the data difficult since reactivity may not be relevant to the epitopes of the BHRF1 protein. It may be due to antibodies to minor antigen populations, such as denatured antigens. These denatured antigens were identified by Rodda *et al.* (1996) to recognise closely related or overlapping epitopes in a protein.

Liu *et al.* (1998) reported that IgG and IgA antibodies to full-length BHRF1 protein were detected in 61.3% of NPC patients. The reactivity result was stronger in IgG but both the specificity of IgA and IgG were good. Results of our study indicate that there are a few predominant antigenic epitopes of the full-length BHRF1 protein. Liu *et al.* (1998) report was based on the used of the full-length BHRF1 protein as antigen in Western blotting. It was shown that Western blotting with polypeptide fragments of the intact protein as an antigen can recognise assembled, discontinuous epitopes, which could not be exhibited in synthetic peptides (Kelly *et al.*, 1995). Synthetic peptides can only mimic linear epitopes.

Antibodies are extremely sensitive to the conformation of a protein antigen. Identical amino acid sequences do not always necessarily identical antigenic characteristics in the synthetic peptide and the intact protein. Furthermore, the short synthetic peptides may not be able to bind the antibody with sufficient specificity. The forms of antigens may vary in differences in structure between the peptides and intact protein (e.g. post-translational modification of the protein) or steric hindrance of binding of antipeptide antibodies (e.g. peptide sequence is masked in the protein structure). Flexibility constraints also causes the antibodies to recognise a full-length protein but not the peptide structure (Benjamin *et al.*, 1984; Leinikki *et al.*, 1993).