ASSOC. PROF .DR. SHAHID HASSAN

(USM SHORT TERM GRANT –304/PPSP/6131223)

"DIAGNOSIS OF NASOPHARYNGEAL CARCINOMA BY DNA AMPLIFICATION OF EPSTEIN-BAR VIRUS GENOMES IN TISSUE OBTAINED BY BIOPSY AND FINE-NEEDLE ASPIRATION"

USM J/P- 06

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN
CANSELORI
UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik: Assoc. Prof. Dr.Shahid Hassan

Nama Penyelidik-Penyelidik Lain (Jika berkaitan) : Yoke-Yeow Yap
M. Ravichandran
Melissa Li-Ann Chan

2) Pusat Pengajian/Pusat/Unit : School of Medical Sciences (PPSP), USM

3) Tajuk Projek: **Diagnosis of nasopharyngeal carcinoma by DNA amplification of Epstein-Bar Virus genomes in tissue obtained by biopsy and fine-needle aspiration**

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman di antara 100 – 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).

ABSTRACT

(English version)

Background

Nasopharyngeal carcinoma (NPC) is common in Malaysia but diagnosis is sometimes delayed for non-representative biopsy, submucosal disease and occult primaries - increasing morbidity and mortality. Epstein-Barr virus (EBV) is associated with all types of NPC and DNA in tumor cells is detectable by polymerase chain reaction (PCR). EBV products EBNA1, EBNA2 and LMP1 are implicated in oncogenesis and is detectable in nodal tissue. However no similar study has been done in Southeast-Asia with adequate sample.

Objectives

This study evaluates the validity and reliability of detecting EBV genes in biopsy and FNAC tissue in NPC by PCR.

Methodology

Tissue from 72 nasopharyngeal biopsies were collected from consented patients. 36 were positive and 36 negatives served as controls. Tissue from 70 fine-needle aspirations were similarly obtained. 35 belonged to NPC-positive patients, and 35 of other pathologies served as controls.

DNA was extracted, amplified with forward and reverse primers for EBNA1, EBNA2, LMP1 genes and human â-actin gene as control, and detected by electrophoresis. Cloned DNA from B95-8 cell line served as positive control.

Histopathological-proven primary tumour and clinico-pathological criteria for neck nodes (clinically suspicious neck node with histopathologically-confirmed primary tumour) were used as gold standard.

Results

35/36 positive nasopharyngeal biopsies and 35/36 negatives contained sufficient DNA. EBNA1 gene was detected in 34/35 positive specimens but were undetected in the controls. EBNA2 gene was detected in 31/35 positive specimens and in 2/35 controls. LMP1 was detected in 32/35 positive specimens and in 4/35 controls. ($P > 0.05$ by McNemar_s test - - i.e. no significant difference from histopathology.) EBNA1 has the best sensitivity (97.1%) and specificity (100%) (Kappa = 0.97). One patient in the control group was positive for EBV DNA and developed NPC 1 year later. Another patient with obvious nasopharyngeal tumour was negative on the 1st biopsy and confirmed on repeat biopsy 2 weeks later, but EBV DNA was detected in both specimens.

35/36 metastatic NPC specimens contained sufficient DNA and one was excluded due to presence of second primary. EBNA1 gene was detected in 30/34 nodes and 1/34 controls. EBNA2 gene was detected in 29/34 nodes and none of the controls. LMP1 gene was detected in 30/34 nodes and in 2/34 of controls. ($P > 0.05$ by McNemar_s test _ i.e. no significant difference from clinico-pathological criteria for neck metastasis). A cut-off point of >0/3 genes offers the highest sensitivity (97.1%) and specificity (94.1%) (Kappa = 0.91).

All histological types of NPC contained EBV DNA.

Conclusion

EBV DNA detection is reliable and accurate in diagnosing NPC. On par with histopathology in detecting primary tumours, it also predicts the development of NPC. On par with clinico-pathological criteria in detecting metastatic NPC, it is superior to fine-needle cytology and can suggest NPC in occult primaries.

ABSTRACT
(Bahasa Melayu version)

Latarbelakang
Karsinoma nasofaring (NPC) adalah kerap di Malaysia tetapi diagnosanya seringkali tertunda - disebabkan biopsi yang tidak tepat, penyakit submukosa, dan _occult primary_ _ menyebabkan morbiditi dan mortality meningkat. Virus Epstein-Barr (EBV) adalah berkait dengan semua jenis NPC dan DNA dalam sel-sel kanser boleh dikesan dengan tindakbalas rantai polymerase (PCR). Hasil-hasil EBV EBNA1, EBNA2 dan LMP1 telah diimplikasikan dalam onkogenesis dan boleh dikesan dalam tisu noda limfa. Walaubagaimanapun tiada kajian yang serupa pernah dijalankan di Asia Tenggara dengan saiz contoh yang memadai.

Objektif
Kajian ini menilaikan kejituan dan keberkesanan pengesanan gen EBV dalam tisu biopsi dan FNAC dalam NPC.

Kaedah
Tisu dari 72 biopsi nasofaring telah diambil dari pesakit-pesakit yang memberi keizinan. Antaranya 36 positif dan 36 negatif dijadikan kawalan. Tisu dari 70 aspirasi-jarum-halus diambil juga. Antaranya 35 diambil dari pesakit NPC dan 35 dari penyakit lain yang dijadikan kawalan.

DNA telah diekstrak, diamplifikasi dengan primer hadapan dan belakang bagi gen-gen EBNA1, EBNA2, LMP1 dan juga gen â-actin manusia sebagai kawalan, dan dikesan dengan elektroforesis. DNA yang diklon dari kultur sel B95-8 dijadikan kawalan positif.

Keputusan
35/36 biopsi nasofaring positif dan 35/36 negatif mengandungi DNA yang mencukupi. Gen EBNA1 telah dikesan dalam 34/35 spesimen positif dan tiada dikesan dalam spesimen kawalan. Gen EBNA2 dikesan dalam 31/35 spesimen positif dan dalam 2/35 kawalan. LMP1 dikesan dalam 32/35 spesimen positif dan 4/35 kawalan. (Nilai P > 0.05 dengan ujian McNemar_s _ menunjukkan tiada perbezaan ketara disbanding dengan histopatologi.) EBNA1 menunjukkan sensitiviti (97.1%) dan spesifisiti (100%) yang terbaik (Kappa = 0.97). Seorang pesakit dalam kumpulan kawalan yang EBV DNA positif telah mendapat NPC 6 dalam masa 1 tahun kemudian. Seorang pesakit lagi mempunyai ketumbuhan ketara dalam nasofaring tetapi biopsi pertamanya negatif manakala biopsi 2 minggu kemudian positif, tetapi DNA EBV telah dikesan dalam kedua-dua specimen.

35/36 spesimen NPC metastatik mengandungi DNA yang mencukupi dan satu diketepikan akibat ketumbuhan kedua. Gen EBNA1 dikesan dalam 30/34 noda dan 1/34 kawalan. Gen EBNA2 dikesan dalam 29/34 noda dan tiada dalam kawalan. Gen LMP1 dikesan dalam 30/34 noda dan 2/34 kawalan. (P > 0.05 dengan ujian McNemar _ iaitu tiada perbezaan ketara disbanding dengan kriteria kliniko-patologi.) Nilai _cut-off_ >0/3 gen memberi sensitivity (97.1%) dan spesifisiti (94.1%) yang terbaik (Kappa = 0.91).

Kesemua jenis NPC mengandungi DNA EBV.

Kesimpulan
Pengesanan DNA EBV adalah jitu dan berkesan dalam mendiagnosa NPC. Ia adalah setara dengan histopatologi dalam pengesanan kanser primari dan juga boleh menjangka terjadinya NPC. Setara dengan kriteria kliniko-patologi dalam mengesan NPC metastatic, ia lebih baik daripada sitologi jarum-halus dan boleh mencadangkan NPC dalam keadaan *occult primary*.

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
Karsinoma nasofaring	Nasopharyngeal Carcinoma
Ekstraksi DNA	DNA extraction
Genom EBV	EBV genome
Tindakbalas rantaian polimerase	Polymerase chain reaction (PCR)
Diagnosis	Diagnosis
.....	.....
.....	.....

5) Output Dan Faedah Projek

(a) Penerbitan (termasuk laporan/kertas seminar)
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

Publications

i. **Research Bulletin Malaysia. (Editorial).** A breakthrough in NPC diagnosis-From conventional biopsy to EBV Amp Kit. Vol –No.2/3 August/Disember 2004 (copy attached)

ii. **Pakistan Journal of Otolaryngology (Editorial).** Conventional biopsy to EBV Amplification kit in quick diagnosis of nasopharyngeal carcinoma. 2004, 20: 38-39 (copy attached)

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Presentations

i. Diagnosis of nasopharyngeal carcinoma by DNA amplification of EBV genomes in nasopharyngeal biopsy and fine needle aspiration of neck nodes, in 10th Asia-Oceania ORL-HNS Congress held in KL (Feb 2004)

ii. EBV genome amplification by PCR method, tissue obtained as biopsy from nasopharynx and the aspirate from neck mass. Research presentatation, School of Medical Sciences, USM (Nov 2004)

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(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten.
(Jika ada dan jika perlu, sila guna kertas berasingan)

i. Based on this research is a quick diagnostic kit called ***Ez EBV Amp- A DNA-based diagnostic kit for the detection of Epstein-Barr virus in Nasopharyngeal Carcinoma***. This kit is being patented. This is an innovation which has been received with surprised by all the participants of Asia-Oceania Congress and many of them have contacted us and shown their interest to use this kit in cases with problems of diagnosis. We intented to present this kit in a number of future conferences once the kit is commercially available.

ii. Kit has been used in Hospital USM in difficult cases of diagnosis with encouraging result.

iii. Ez EBV Amp- A DNA-based diagnostic kit has bwon the number of awards including the Gold medal at national and international level
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(c) Latihan Gunatenaga Manusia

i) Pelajar Siswazah: .....
This research was also utilized as a dissertation topic for one of the postgraduate MMed candidate in ORL-HNS during 2003/04 as under:
Diagnosis of Nasopharyngeal Carcinoma bty DNA Amplification of Epstein-Barr Virus Genomes in Tissue obtained by biopsy and fine Needle Aspiration (Copy attached)
Candidate: Dr Yap Yoke Yeow
Principal Supervisor: Assoc. Prof Dr Shahid Hassan
Co-Supervisor: Assoc. Prof Dr Ravichandran
.....

The dissertation was given excellence remarks with the recommendation to publish the work in high index medical journal, which it it send already to British Journal of Laryngology and Otology (copy attached).

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ii) Pelajar Prasiswazah: .....

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iii) Lain-Lain : .....

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6. Peralatan Yang Telah Dibeli:

No equipment as such was bought, only chemical were purchased for this research which was fully utilized.

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UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

.....Layak menerima kegiatan ini dijalankan.

.....Hasil penyelidikan emas diharapkan

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T/TANGAN PENGERUSI
J/K PENYELIDIKAN
PUSAT PENGAJIAN

PROFESSOR ABDUL AZIZ BABA
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Diagnosis of Nasopharyngeal Carcinoma by DNA Amplification of EBV Genomes in Nasopharyngeal Biopsy and Fine-Needle Aspiration of Neck Nodes

Shahid Hassan, Yap Y.Y, Ravichandran M.

Abstract

Background
Nasopharyngeal carcinoma (NPC) is common in Malaysia but diagnosis is sometimes delayed for non-representative biopsy, submucosal disease and occult primaries - increasing morbidity and mortality. Epstein-Barr virus (EBV) is associated with all types of NPC and DNA in tumor cells is detectable by polymerase chain reaction (PCR). EBV products EBNA1, EBNA2 and LMP1 are implicated in oncogenesis and is detectable in nodal tissue. However no similar study has been done in Southeast-Asia with adequate sample.

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EBV DNA detection is reliable and accurate in diagnosing NPC. On par with histopathology in detecting primary tumours, it also predicts the development of NPC. On par with clinico-pathological criteria in detecting metastatic NPC, it is superior to fine-needle cytology and can suggest NPC in occult primaries.

1 Introduction

Nasopharyngeal carcinoma (NPC) is a potentially curable disease if detected early. However, many pitfalls in the detection of NPC may delay diagnosis, increase morbidity and mortality, and worsen the prognosis of the disease. These include late presentation, late referral to the ENT surgeon, difficulties in interpreting biopsy material and the entity of the 'occult primary'. This study evaluates DNA amplification by polymerase chain reaction (PCR) of Epstein-Barr virus (EBV) genes and its role in areas of dilemma in the diagnosis of NPC.

1.1 Background

1.1.1 Carcinoma of the nasopharynx

Nasopharyngeal carcinoma (NPC) is an epithelial tumour that is rare in most parts of the world - the incidence rate being less than 1 per 100,000 persons per year. It has a distinct geographical and population pattern, gravitating towards China, Southeast Asia, Africa, Canada, Alaska and Greenland Eskimos. The highest incidence is observed in the Cantonese-speaking Chinese of Guangdong province in South China, with an incidence of 30-50 per 100,000 persons per year, and high frequencies are observed similarly in emigrant southern Chinese populations in South-east Asia.

NPC is the commonest head and neck cancer in Malaysia. In Peninsular Malaysia, the incidence of NPC is 365 cases per year or 5.4 per 100,000 population (Prasad and Rampal, 1992). This disease affects mostly the Chinese (14.6 per 100,000), followed by the Malays (1.3 per 100,000) and least of all, the Indians (0.5 per 100,000). The highest incidences fall in the states of Selangor (26.9 per 100,000), Pahang (12.3 per 100,000) and the Federal Territory (10.7 per 100,000). The incidence rate rises after the age of 20 and plateaus at 40 to 59 years without any further rise with increasing age. The age-adjusted male:female ratio is 2.8 to 1.

NPC constitutes 85% of malignant tumours of the nasopharynx, followed by lymphomas and other rare tumours such as the adenocarcinoma, adenoid cystic carcinoma and malignant melanoma (Watkinson *et al.*, 2000). NPCs are classified by the World Health Organization (WHO) into three types:

1. **Type I - Keratinising squamous cell carcinoma (SCC)**, seen on light microscopy and subdivided into well-, moderately-, and poorly-differentiated;
2. **Type II - Nonkeratinizing carcinoma (NK)** which is undifferentiated and shows squamous origins only on electron microscopy - and immunohistochemistry; and
3. **Type III - Undifferentiated carcinoma (UC).**

All types of NPC are now regarded as variants of the squamous cell carcinoma. However, keratinising SCC (WHO Type I) is uncommon and the well-differentiated subtype is extremely rare. In a study of 1800 cases of NPC in Malaysia, (Pathmanathan *et al.*, 1995) found 17% to be WHO type I and only 0.2% to be well-differentiated.

1.1.2 Epstein-Barr virus in NPC

The Epstein-Barr virus (EBV) is a double-stranded DNA virus of the *Herpesviridae* family, first described by Epstein *et al.* in 1964. Apart from their infective illnesses, EBV is strongly associated with Burkitt's lymphoma, B-cell lymphoma, Hodgkin's disease, T cell lymphomas and NPC. It may sometimes be found in lymphoepithelial carcinomas of the stomach, salivary gland, lung and thymus (Allen, 1999, Kanegane *et al.*, 2002), and also with oral cancers in Japan (Shimakage *et al.*, 2002)

Old *et al.* first described the serological relationship between EBV and NPC in 1966 and it has been subsequently established that patients with NPC have elevated antibody levels against various EBV-associated antigens, correlating with stage and tumour burden. NPC cells were shown by Klein, *et al.* (1974) to contain multiple copies of the EBV genome. EBV genomes and associated antigens have consistently been found in undifferentiated and well-differentiated NPC (Raab-Traub *et al.*, 1987). The strong and consistent association of EBV with NPC suggests that it may have an oncogenic role.

Pathmanathan, *et al.* (1995) using polymerase chain reaction amplification of cDNA to detect EBV transcription for EBNA1, LMP1 and LMP2 genes showed that all types of NPC, regardless of histology or differentiation, contain episomal EBV genomes and express specific EBV genes. Moreover EBV DNA was found in the preinvasive state of NPC. Similarly Huang and Lo (1999) described the presence of EBV DNA in all forms of NPC as well as the precursor state of Nasopharyngeal Intra-Epithelial Neoplasia Type III.

Figure 1 - Map of the complete Epstein-Barr virus genome showing the PCR target zones for EBNA1, EBNA2 and LMP1 genes

1.1.3 Polymerase Chain Reaction (PCR)

PCR can be used to detect and amplify specific nucleic acid sequences of a virus. Targeted amplification of nucleic acid sequences provides dramatic increases in the number of copies as well as equivalent reduction in the complexity of the nucleic acid to be probed. Also, the exponential amplification of PCR catalyzed by this biochemically simple cyclical process requires less than minutes per cycle obviating the need for *in vitro* cell culture of a virus which may take weeks to months. These aspects of PCR allow ready detection of a single virus particle especially when a specific host cell cannot be cultured and in latent infections where active viral replication is substantially attenuated thereby obviating procedures requiring the detection of proteins (Kwok and Sninsky, 1999). Such is the case of the Epstein-Barr virus in NPC which establishes a latent infection in the epithelial cells of NPC. Needle punch biopsies and aspirates of various types have been shown to provide sufficient material for PCR analysis (Kwok and Sninsky, 1989).

1.2 Problems in the diagnosis of NPC

1.2.1 The 'normal nasopharynx'

A nasopharyngeal mass seen on examination can be biopsied under direct vision, utilizing a flexible or rigid endoscope, and the diagnosis is made quite easily. However, patients suspected clinically as having NPC but whose nasopharynx appears normal, will need deep biopsies taken from multiple sites and both Fossae of Rossenmüller. These include patients with persistent unilateral serous otitis media, metastatic nodal disease, raised EBV titres, or radiological evidence consistent with NPC. Sometimes, serial and multiple biopsies need to be undertaken before a diagnosis is reached (Woo, 1999). A significant proportion (13.3%) have occult primaries at presentation and the primary tumour may remain undetected even after repeated biopsies of the nasopharynx (Prasad *et al.*, 1983). Causes of false negatives are non-representative biopsies, submucosal disease, and failure to recognize individual malignant cells or small clumps of tumour (Allen, 1999).

1.2.2 Cytological evaluation

Fine-needle aspiration (FNA) of nodal disease in NPC is a useful tool and can spare the patient from open biopsy which worsens the long-term prognosis considerably (Cai *et al.*, 1983). It can make the diagnosis of metastatic undifferentiated or differentiated squamous carcinoma, and other carcinomas (e.g. adenocarcinoma, small cell carcinoma, sinonasal carcinoma). It may even suggest nasopharyngeal carcinoma as the origin when the presence of clusters of cohesive tumour cells, medium-sized oval vesicular nuclei, prominent nucleoli, pale cytoplasm, mitotic figures and intermingling mature lymphocytes are seen (Chan *et al.*, 1988). With the help of immunohistochemical staining, carcinomas can be differentiated from lymphomas and granulomatous diseases. However, FNA has limitations, which include inadequate or non-representative specimen, poor cellular preservation and an inexperienced cytopathologist (Chang and Chan, 1999). A study in Singapore on 123 fine-needle aspirates of neck masses (Kaur *et al.*, 1993) had a diagnostic accuracy of 82.6% and inadequacy rate of 12.2%. . In a retrospective study of 57 cases of metastatic carcinoma of the neck with unknown primary found that only in 19 cases (33.3%) could the primary site be found with a rigorous diagnostic algorithm of lymph node biopsy, rigid panendoscopy with systematic biopsies of suspect regions as well as blind biopsies of endoscopically inconspicuous regions, including the tongue base and nasopharynx and bilateral tonsillectomy (Haas *et al.*, 2002). Chang & Chan (1999) proposes the detection of EBNA (Epstein-Barr virus nuclear antigen) or in-situ hybridisation of EBER (Epstein-Barr virus-encoded RNA) on histological and cytological material as 'a very useful adjunct in diagnostic pathology', especially when conventional immunohistochemical techniques are unhelpful.

1.3 Review of the literature

Various studies have been done to ascertain the feasibility of detecting EBV DNA and RNA in the fine-needle aspirate of metastatic nodal disease of NPC. A comparison of their results is shown in the Table 1 below.

Table 1 - Comparison of various studies done to detect EBV genes in the fine-needle aspirate of metastatic nodal disease in NPC

Author	Year	Country	NPC patients	Controls	Sensitivity %	Specificity %
Ohshima *et al.*	1991	Japan	4/4	5/32	100	78.1
Walter *et al.*	1992	USA	15/18	0/17	88.9	100
Feinmesser *et al.*	1992	Canada	9/9	2/137	100	98.5
Pacchioni *et al.*	1994	Italy	7/7	0/18	100	100
MacDonald *et al.*	1995	Canada	5/5	2/41	100	95.1
Smith *et al.*	1995	USA	1/1	-	-	-
Akao *et al.*	1996	Japan	3/3	-	-	-
Lee *et al.*	2000	Taiwan	10/10	0/20	100	100

These studies have all been conducted outside of Southeast Asia. Their sample sizes have been small, ranging from 1 to 18 patients with confirmed NPC. Although they demonstrate a consistently high sensitivity (88.9 – 100%) and specificity (78-100%), further study is needed to validate these findings with a larger sample size before PCR can be used as a diagnostic tool.

In the eight studies reviewed above, two studies were done using Southern blot as the detection method (Ohshima *et al.*, 1991, Smith *et al.*, 1995) and Ohshima *et al.*(1991) found that in cancers other than the lymphoepithelioma, only PCR could reliably detect EBV DNA. Three other studies utilized PCR as the detection method (Feinmesser *et al.*, 1992b, Macdonald *et al.*, 1995, Walter *et al.*, 1992). In-situ hybridization on tissue obtained from lymph nodes were used in another three studies (Akao *et al.*, 1996, Lee *et al.*, 2000, Pacchioni *et al.*, 1994).

While Lee *et al.* (2000) detected EBERs to indicate the presence of EBV, the other seven studies used EBV DNA. Feinmesser *et al.* (1992b) used probes for EBNA1 and EBNA2 with much success.

In three of these studies there were patients whose primary tumour was unknown at the time of sampling by FNAC but neck node tissue tested positive for EBV DNA. 2 out of 2 patients in the study by Feinmesser *et al.* (1992b), 1 out of 2 in the study by MacDonald *et al.* (1995) and 1 out of 1 in the study by Smith *et al.* (1995) subsequently developed overt NPC which were confirmed histologically. However, in none of these studies has the accuracy of detecting different genes been compared nor has the value of detecting multiple genes in combination been considered.

Walter *et al.* (1992) concludes that their study 'demonstrates the utility of EBV detection by the polymerase chain reaction in the evaluation of patients with metastases to neck nodes from occult primary carcinomas.' Pacchioni *et al.* (1994) in their study on undifferentiated NPC (UNPC) makes a similar conclusion that is 'detection of EBV in cervical metastatic adenopathy may be successfully used to identify the presence of occult UNPC.' MacDonald *et al* (1995) says their results 'demonstrate the utility of NPC-diagnostic EBV gene amplification in FNA samples of neck metastases and suggest that the presence of the EBV genome in FNA samples of neck nodes is predictive of the presence of NPC.' Moreover, Feinmesser *et al.* (1992) adds that 'the presence of EBV in metastases from an occult primary tumor is predictive of the development of overt nasopharyngeal carcinoma.'

It is the objective of this study therefore to confirm the value of detecting EBV DNA as a diagnostic tool in NPC in this region. Using a larger-sized sample, this study will also attempt to compare the accuracy of EBV DNA detection of three different genes, and its value when taken in combination. Chapter 2 details the general and specific objectives of this study. In Chapter 3, the exact methodology of patient selection, specimen collection, stratification, laboratory analysis and statistical method is outlined. In Chapter 4, the results of this study are presented together with its validity data and statistical

significance. Finally, in chapter 5, the importance and significance of the results are discussed and recommendations are presented in Chapter 6.

2 Objectives

2.1 General objectives

2.1.1 To evaluate the amplification of EBV DNA in tissue as a diagnostic tool for nasopharyngeal carcinoma

2.1.2 To establish Malaysian data regarding the amplification of EBV DNA in tissue of nasopharyngeal carcinoma

2.1.3 To compare the detection rate of various EBV genes individually and in combinations, in the tissue of nasopharyngeal carcinoma

2.2 Specific objectives

2.2.1 To establish the validity of EBV DNA detection of three EBV genes (EBNA1, EBNA2 and LMP) in postnasal space biopsy and fine-needle aspirate of neck masses in NPC

2.2.2 To determine the diagnostic accuracy of EBV DNA detection of three paired-gene combinations in postnasal space biopsy and fine-needle aspirate of neck masses in NPC

2.2.3 To determine the optimal diagnostic cut-off value for the total number of genes detected in postnasal space biopsy and fine-needle aspirate of neck masses in NPC

2.2.4 To compare the accuracy of FNAC and PCR in the detection of metastatic nodal disease

2.2.5 To ascertain the association between EBV genes with histological type

3 Methodology

This is a study to evaluate the validity of DNA amplification of various Epstein-Barr viral genes compared to histopathological examination (HPE) as the gold standard.

3.1 Population, period and place of study

3.1.1 Population: Patients from the Otorhinolaryngology clinic of Hospital USM, Kelantan and Ear, Nose and Throat Clinic of Hospital Pulau Pinang

3.1.2 Period of study: July 2002 to September 2003

3.1.3 Place of study: Otorhinolaryngology Clinic of Department of Otorhinolaryngology and Head & Neck Surgery, USM, Ear, Nose, and Throat Clinic of Hospital Pulau Pinang, Microbiology Laboratory of Department of Microbiology, Histopathology Laboratory of Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia (USM).

3.2 Sample size

3.2.1 Active

71 tissue specimens positive for nasopharyngeal carcinoma were selected for PCR analysis (36 primary tumour biopsy and 35 nodal metastasis fine-needle aspirates) from patients who fulfilled the inclusion criteria. These specimens were grouped as

1. Group N (36) – proven NPC, postnasal biopsy of primary tumour in the nasopharynx
2. Group F (35) – proven NPC, fine-needle aspirate of metastatic neck node

3.2.2 Control

71 tissue specimens negative for nasopharyngeal carcinoma were selected (36 postnasal space biopsy and 35 neck mass fine-needle aspirates) from patients who fulfil the inclusion and exclusion criteria.

These specimens were grouped as:

1. Group A (36) – proven non-NPC, postnasal biopsy
2. Group B (35) – proven non-NPC, neck mass fine-needle aspirate

3.2.3 Sample size calculation

Sample size was derived with a Power and Sample Size Program®†, based on the confidence limit of 0.95, type I error [α] of 0.05, power of study [1-β] of 0.9, type II error [β] of 0.1, odds ratio [ψ] of 171, and correlation coefficient [ϕ] (arbitrary) of 0.5.

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The required sample size of 34 pairs for each group was reached (34 for postnasal space biopsy and 34 for neck mass FNAC.)

3.3 Patient recruitment

The number of patients recruited into this study was less than the number of specimens as more than one specimen may have been procured from a single subject. Postnasal biopsy and fine-needle aspiration were performed in patients with a primary tumour and neck metastasis. Fine-needle aspirates were taken from each neck node if multiple clinically suspicious neck nodes existed.

3.3.1 Inclusion criteria

- Patient with suspected nasopharyngeal carcinoma, with or without neck metastasis, undergoing postnasal space biopsy
- Patient with neck mass undergoing fine-needle aspiration – confirmed cytologically or histopathologically to be a head and neck cancer other than the lymphomas, a benign disease or normal nodal tissue.

3.3.2 Exclusion criteria

- Patients with more than one head and neck malignancy,
- Patients who have been subjected to radiotherapy or chemotherapy, or
- Debilitated patients who are unable to tolerate biopsy, fine-needle aspiration or surgery.

3.3.3 Consent

Informed consent was obtained before enrolling a patient into the study. He or she would be made to understand the nature of this study as explained in the Information Sheet (Appendices B and C) and must voluntarily agree to be part of this research. A translator was provided and consent was taken in the presence of an impartial witness when the patient could not understand English or Malay.

3.3.4 Proforma

The patients' data were recorded in a Proforma (Appendix D) designed for this study. The data includes:-

- Patient biodata
- Inclusion and exclusion criteria
- Tissue collection – type and date
- Tumour TNM staging
- Histopathological type and grade
- Laboratory analysis and results

3.4 Specimen collection

Tissue was taken pre-emptively during routine diagnostic biopsy of the nasopharynx or fine-needle aspiration of neck masses. As the diagnosis was not known at the time of biopsy, tissue was stored and subsequently classified into the various groups (N, F, A, B) based on the histopathology report. Patients in the active groups (N and F) may have had one or more specimens taken as part of routine diagnostic investigations - a biopsy of the primary tumour of the nasopharynx and/or a fine-needle aspiration of the metastatic neck node for each clinically suspicious neck node present. Patients in the control groups (A, B) may have had one or more tissue specimens taken from them – either from the nasopharynx or from a palpable neck node of any pathology other than NPC or lymphoma.

3.4.1 Biopsy of the nasopharynx

Informed consent was obtained and a biopsy from an obvious growth or the Fossa of Rossenmüller under visualisation was performed with a rigid or flexible endoscope in the standard way (Fig. 8). The main bulk of tissue was placed in formaldehyde to be sent for HPE and a small segment of tissue, 1-2mm in size was placed in a 1.5mL tube and stored for later analysis pending histopathological confirmation . If the histopathological examination (HPE) proved to be positive for NPC, the patient was recruited into the study under group N and if negative the specimen was used as a control in group A.

3.4.2 Fine-needle aspiration of neck masses

Informed consent was taken for each patient and fine-needle aspiration was performed for neck masses using a 23G needle, 20cc syringe and needle biopsy carrier in the standard way (Fig. 9). The main bulk of aspirate was smeared onto slides for cytological examination and the remainder put into a 1.5mL tube and stored for later DNA analysis. If the specimen belongs to a patient with confirmed nasopharyngeal carcinoma on biopsy of the nasopharynx, it is classified under group F. Otherwise the specimen was classified under group B unless cytological examination shows lymphoma, in which case it is excluded (because certain lymphomas are known to contain EBV and can be diagnosed by FNAC alone)

3.4.3 Storage and transport

Tissue specimens in 1.5mL tubes were stored in a freezer at the clinic temporarily and then transported in an ice-box to the -20°C freezer at the soonest possible time.

3.5 Laboratory analysis

3.5.1 Histopathological examination (HPE)

All specimens collected were sent for routine histopathological and cytological examination. The diagnosis obtained from the pathologist confirmed nasopharyngeal carcinoma or otherwise and determined how they were classified (N,F,A,B). PCR results were compared to the histopathological diagnosis as the gold standard.

3.5.2 PCR Analysis

All 138 tissue specimens with sufficient DNA (human β-actin gene positive) from the active and control groups were subjected to PCR analysis. This method involves three main steps:

1. Extraction of total DNA from tissue specimens,
2. Amplification of total DNA by PCR, and
3. Detection of EBV DNA by agarose gel electrophoresis.

PCR was performed at the Department of Microbiology, USM. They were analysed for the presence of three EBV genes – EBNA1, EBNA2 and LMP1 – and the human β-actin gene to ensure sufficient amounts of DNA extraction.

3.5.2.1 Primer design

Primers are short oligonucleotides made of T,A,G and C nucleotides used by DNA polymerase to initiate synthesis of new complementary strands of DNA. Forward and reverse primers were designed for the Epstein-Barr virus genes EBNA1, EBNA2 and LMP and also for the human β-actin gene (Table 2).

Primers were custom-made according to specification by a commercial company[1] (Appendix G).

3.5.2.2 EBV DNA positive control

EBV DNA positive control was derived from a B95-8 cell line[2]. EBV DNA from the B95-8 cell line was amplified with *Taq* polymerase PCR using primers designed specifically for EBNA1, EBNA2 and LMP1. The PCR products were then cloned into PCR cloning vector TOPO 2.1 (Invitrogen) (Fig. 10). The positive clones were selected by PCR screening. The presence of EBV gene was further characterized by restriction analysis. The positive EBV clones were then used as positive control in each PCR analysis.

3.5.2.3 Extraction of tissue DNA

DNA from the tissue samples were extracted using the Nucleospin® tissue DNA extraction kit (Fig. 11) (Appendix G). Approximately 30mg of tissue was digested in lysis buffer containing Proteinase K at 56°C overnight (Fig. 12, 13). The digested tissue sample was passed through a silica column (Fig. 14). Since DNA has a high affinity for silica, it binds to it, and pure DNA was eluted from the column using a low-salt buffer.

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[1] Alpha DNA®

[2] Courtesy of Dr. Peh, University Malaya

3.5.2.4 Amplification of EBV DNA by PCR

1) Forward and reverse primers for EBNA1, EBNA2, and LMP genes of the Epstein-Barr virus were used in the polymerase chain reaction. Probes for the human β-actin gene were also used for every set to confirm sufficient quantity of DNA available for PCR amplification.

3.5.2.5 Detection of PCR product by electrophoresis

The PCR product obtained was run on 1% agarose gel containing ethidium bromide at 10 volts/cm (Fig. 16, 17, 18). Electrophoresis separates DNA molecules based on size and charge in an electric field. Agarose is a seaweed that, when boiled, forms a matrix of interlocking fibres and pores. The PCR product is loaded into wells at one end and negatively charged DNA migrates towards the anode. Smaller molecules will migrate at a higher velocity, resulting in a gradient based on molecular size. Ethidium bromide binds to DNA and serves as a DNA staining agent. Using an image analyser, DNA bands will fluoresce under ultraviolet light and an image is photographed using the image analyzer. The expected product size is compared with a marker (Appendix G).

3.6 Data collection

All data were stored digitally using Microsoft Excel 2000 spreadsheet as a database.

3.7 Statistical analysis

All statistical analyses were performed using the proprietary software MedCalc® ver 7.2.0.2†.

3.7.1 Specific Objective 1 : To establish the validity of EBV DNA detection of three EBV genes (EBNA1, EBNA2 and LMP) in postnasal space biopsy and fine-needle aspirate of neck masses, in NPC

3.7.2 Specific objective 2 : To determine the diagnostic accuracy of EBV DNA detection of three paired-gene combinations in postnasal space biopsy and fine-needle aspirate of neck masses, in NPC

The detection rate for three EBV gene-pairs for active and control groups were tabulated, i.e. for

- EBNA1 & EBNA2
- EBNA1 & LMP1
- EBNA2 & LMP1

3.7.3 Specific objective 3: To determine the optimal diagnostic cut-off value for the total number of genes detected in postnasal space biopsy, in NPC.

The detection rate for using different cut-off points total number of genes for active and control groups were tabulated, i.e. for

- Cut-off point – 1 or more genes (>0)
- Cut-off point – 2 or more genes (>1)
- Cut-off point – 3 genes (>2)

3.7.4 Specific objective 4: To compare the accuracy of FNAC and PCR in the detection of metastatic nodal disease

The results of FNAC in clinically positive neck nodes of patients with proven primary tumour of NPC were compared with the detection of EBV genes by percentages.

3.7.5 Specific objective 5: To ascertain the association between EBV genes with histological type.

Detection rates for each gene were calculated for each histological type – Type I, II and III – according to the WHO classification.

4 Result

4.1 Subjects

4.1.1 Postnasal space biopsy

In the period of study, tissue from a total of 86 postnasal space biopsies were collected and stored.

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Upon histopathological confirmation of the 86 postnasal biopsies, 36 were positive for nasopharyngeal carcinoma and 50 were negative (of which the first 36 were selected for PCR analysis). During PCR analysis for human β-actin gene, one specimen from group N and one from group A was negative and duly excluded from the study. The final number of specimens was for Group N - 35 specimens and Group A - 35 specimens.
The breakdown of their histopathological/cytological diagnoses is as follows:

- Group N (35)
  - WHO type I – 4 (11.4%)
  - WHO type II – 3 (8.6%)
  - WHO type III – 28 (80%)
- Group A (35)
  - No malignancy – 32 (91.3%)
  - Chronic inflammation -3 (85.7%)

4.1.2 Fine-needle aspirate of neck masses

In the period of study, tissue from a total of 71 fine-needle aspirates were collected and stored. Upon cytological and histopathological confirmation, 36 neck nodes belonged to patients with nasopharyngeal carcinoma (proven by postnasal biopsy) and 35 others had other diagnoses. One specimen from group F was reported as a 'vascular tumor' and thus excluded on the grounds that it may have been a second primary (as per exclusion criteria). During PCR analysis for human β-actin gene, one specimen from group F and one from group B were negative and duly excluded from the study. The final number of specimens was Group F - 34 specimens and Group B - 34 specimens.
The breakdown of their cytological diagnoses is as follows:

- Group F (34)
  - Metastatic carcinoma – 23 (67.6%)
  - No malignancy – 4 (11.8%)
  - No diagnostic material or insufficient for evaluation – 4 (11.8%)
  - Atypical cells – 2 (5.9%)
  - Necrotic debris -1 (2.9%)
- Group B (34)
  - Metastatic CA (10) – from ethmoid CA (2), CA ovary (1), oral CA (3), Undifferentiated parotid CA (2) and unknown primary (2) – (29.4%)
  - No malignancy – 3 (8.8%)
  - Chronic inflammation – 2 (5.9%)
  - Cystic lesion – 1 (2.9%)
  - Lymphoid cells – 1 (2.9%)
  - No diagnostic material or insufficient for evaluation – 17 (50%)

4.2 Individual EBV gene detection for postnasal space biopsy

Table 2 – Validity data for individual EBV gene detection in postnasal space biopsy (N series)

	EBNA1	EBNA2	LMP1
Sensitivity	97.14%	88.57%	91.43%
Specificity	100.00%	94.29%	88.57%
Positive predictive value	100.00%	93.94%	88.89%
Negative predictive value	97.22%	89.19%	91.18%
Likelihood ratio	infinity	15.50	8.00
95% Confidence interval	-0.96% to 1.43%	-4.68% to 7.66%	-6.25% to 7.92%
X^2	0.0000	0.1667	0.0000
p value	1.000 (>0.05)	0.6831 (>0.05)	1.0000 (>0.05)
Kappa statistic	0.971	0.829	0.800

4.3 Individual EBV gene detection for neck mass fine-needle aspirate

Table 3 - Validity data for individual EBV gene detection in fine-needle aspirate of neck masses (F series)

	EBNA1	EBNA2	LMP1

Sensitivity	88.24%	85.29%	88.24%
Specificity	97.06%	100.00%	94.12%
Positive predictive value	96.77%	100.00%	93.75%
Negative predictive value	89.19%	87.18%	88.89%
Likelihood ratio	30.00	infinity	15.00
95% Confidence interval	-2.42% to 8.22%	0.70% to 8.70%	-4.21% to 9.23%
X^2	1.5000	4.1667	0.5714
p value	0.2207 (>0.05)	0.0412 (>0.05)	0.4497 (>0.05)
Kappa statistic	0.853	0.853	0.824

4.4 Paired gene detection in postnasal space biopsy

Table 4 - Validity data for three paired-gene combinations in postnasal space biopsy (N series)

	EBNA1 + EBNA2	EBNA1 + LMP1	EBNA2 + LMP1
Sensitivity	88.57%	91.43%	88.57%
Specificity	100.00%	100.00%	94.29%
Positive predictive value	100.00%	100.00%	93.94%
Negative predictive value	89.74%	92.11%	89.19%
Likelihood ratio	infinity	infinity	15.50
95% Confidence interval	-1.12% to 5.71%	-1.67% to 4.29%	-4.68% to 7.66%
X^2	2.2500	1.3333	0.1667
p value	0.1336 (>0.05)	0.2482 (>0.05)	0.6831 (>0.05)
Kappa statistic	0.886	0.914	0.829

4.5 Paired gene detection in neck mass fine-needle aspirate

Table 5 - Validity data for three paired-gene combinations in fine-needle aspirate of neck masses (F series)

	EBNA1 + EBNA2	EBNA1 + LMP1	EBNA2 + LMP1
Sensitivity	79.41%	82.35%	82.35%
Specificity	100.00%	97.06%	100.00%
Positive predictive value	100.00%	96.55%	100.00%
Negative predictive value	82.93%	84.62%	85.00%
Likelihood ratio	infinity	28.00	infinity
95% Confidence interval	2.99% to 11.59%	-0.61% to 11.11%	1.81% to 10.14%
X^2	6.1250	3.1250	5.1429
p value	0.0133 (<0.05)	0.0771 (>0.05)	0.0233 (<0.05)
Kappa statistic	0.794	0.794	0.824

4.6 Cut-off value analysis for total number of genes detected in postnasal space biopsy

Table 6 – Validity data for total number of genes detected in postnasal space biopsy (N series)

	>0/3	>1/3	>2/3
Sensitivity	97.14%	91.43%	88.57%

Specificity	88.57%	94.29%	100.00%
Positive predictive value	89.47%	94.12%	100.00%
Negative predictive value	96.88%	91.67%	89.74%
Likelihood ratio	8.50	16.00	infinity
95% Confidence interval	-3.03% to 6.67%	-4.94% to 6.22%	-1.12% to 5.71%
X2	0.8000	0.0000	2.2500
p value	0.3711 (>0.05)	1.0000 (>0.05)	0.1336 (>0.05)
Kappa statistic	0.771	0.857	0.886

4.7 Cut-off value analysis for total number of genes detected in fine-needle aspirate of neck masses

	>0/3	>1/3	>2/3
Sensitivity	97.06%	85.29%	79.41%
Specificity	94.12%	97.06%	100.00%
Positive predictive value	94.29%	96.67%	100.00%
Negative predictive value	96.97%	86.84%	82.93%
Likelihood ratio	16.50	29.00	infinity
95% Confidence interval	-4.83% to 4.83%	-1.58% to 9.66%	2.99% to 11.59%
X^2	0.2500	2.2857	6.1250
p value	0.6171 (>0.05)	0.1306 (>0.05)	0.0133 (<0.05)
Kappa statistic	0.912	0.824	0.794

Table 7 - Sensitivity, specificity and area-under-curve for ROC in cut-off points for FNAC

Criterion	Sensitivity(95%CI)	Specificity(95%CI)	Area under curve
> 0/3	97.1 (84.6- 99.5)	94.1 (80.3- 99.1)	0.956
> 1/3	85.3 (68.9- 95.0)	97.1 (84.6- 99.5)	0.912
> 2/3	79.4 (62.1- 91.3)	100.0 (89.6-100.0)	0.897

4.8 Comparison of FNAC and PCR

In 11 cases of NPC with neck metastasis, PCR was able to detect EBV DNA while FNAC failed to detect malignancy. However in one case, FNAC showed metastatic carcinoma while PCR failed to detect any of the three genes.

Table 8 – Comparison of EBV gene detection and various cytological findings in the fine-needle aspirates of neck nodes in patients with known nasopharyngeal carcinoma.

FNAC for neck nodes in NPC (n=34)	EBNA1	EBNA2	LMP1	Any 1 out of 3 genes
Metastatic CA 67.6% (23)	20/23 (87.0%)	20/23 (87.0%)	19/23 (82.6%)	22/23 (95.7%)
No malignancy (4)	3/4 (75%)	2/4 (50%)	4/4 (100%)	4/4 (100%)
No diagnostic material or insufficient for evaluation (4)	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)
Atypical cells (2)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
Necrotic debris (1)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)
Total (34)	30/34 (88%)	29/34 (85.3%)	30/34 (88.2%)	33/34 (97.1%)

4.9 Comparison of histological type and EBV gene detection by PCR

Of the 35 specimens of NPC postnasal biopsy tissues, 4 were classified as WHO type I (keratinizing squamous cell carcinoma), 3 were WHO type II (non-keratinising squamous cell carcinoma) and the other 28 were WHO type III (undifferentiated carcinoma).
A comparison of the histological type and EBVgene detection by PCR is tabulated below.

Table 9 - Comparison of histological type and EBV genes

	EBNA1	EBNA2	LMP1
WHO type I (4)	4/4	4/4	4/4
WHO type II (3)	3/3	3/3	3/3
WHO type III (28)	27/28	24/28	25/28

5 Discussion

5.1 Significance of results

35/36 positive nasopharyngeal biopsies and 35/36 negatives contained sufficient DNA. EBNA1 gene was detected in 34/35 positive specimens and in none of the controls. EBNA2 gene was detected in 31/35 positive specimens and in 2/35 controls. LMP1 was detected in 32/35 positive specimens and in 4/35 controls. HPE failed to detect malignancy in one patient who had an obvious tumour of the nasopharynx but was only confirmed to be NPC on a second biopsy 2 weeks later. EBV DNA was found in both specimens. Another patient in the control group was positive for EBV DNA and developed NPC within 1 year.

35/36 metastatic NPC specimens contained sufficient DNA and one was excluded due to presence of second primary. EBNA1 gene was detected in 30/34 nodes and 1/34 controls. EBNA2 gene was detected in 29/34 nodes and none of the controls.

5.1.1 Individual EBV gene detection for postnasal space biopsy

In the tissue obtained from postnasal space biopsy, detection of EBNA1 gene is considerably better than that of EBNA2 and LMP1, showing a high sensitivity (97.1%) compared to EBNA2 (88.6%) or LMP1 (91.4%), and an extremely high specificity (100%) compared to EBNA2 (94.3%) or LMP1 (88.6%) as shown in Table 4. All three detection rates have p values above 0.05 on McNemar's test (Table 4) - i.e. there is no significant difference between PCR and HPE. This shows the EBV DNA detection to be on par with the gold standard of histopathological examination. This is supported by the Kappa statistic which shows an excellent inter-test agreement (0.97) for EBNA1 and good agreement (0.8-0.9) for the other two genes (Table 4). Comparing the ROC curves with one another, the results indicate that EBNA1 gene has the highest area-under-curve (0.99) and is significantly different from that of LMP1 ($p<0.05$), and no statistical difference is shown between EBNA2 and LMP1 ($p>0.05$) as shown in Table 5 and 6. EBNA1 gene, showing the highest sensitivity and specificity, Kappa value and area under the ROC curve, makes it the most suitable and specific gene for the diagnosis of NPC in the biopsy tissue of the postnasal space. It is on par with the gold standard of HPE and can provide an adjunct or alternative to diagnosis when it is not forthcoming from histopathological examination.

5.1.2 Individual gene detection for fine-needle aspirate of neck masses

In this study, a clinico-pathological criteria (i.e. clinically suspicious neck node with histopathologically-confirmed NPC of the primary site) is taken as evidence of neck metastasis. In the fine-needle aspirate of neck masses, detection of EBNA1 and LMP1 genes are equally sensitive (88.3%) while EBNA2 is only slightly lower (85.3%) as shown in Table 7. However EBNA2 stands out in specificity (100%) while EBNA1 comes in second (97.1% - 1 false positive) followed by LMP1 (94.1% - 2 false positives). All three detection rates have p values above 0.05 on McNemar's test (Table 4) - i.e. there is no significant difference between PCR and clinico-pathological criteria. The Kappa statistic in this set is consistently high (>0.8) for all three genes, with EBNA1 and EBNA2 showing equal and the highest level (0.853) of inter-test agreement with histopathology (Table 7). Comparing their ROC curves with one another, the results indicate that the detection of EBNA1 has the highest area-under-curve (0.97) and is significantly different from that of EBNA2 and LMP1 ($p<0.05$), but no statistical difference is shown between EBNA2 and LMP1 ($p>0.05$) as shown in Table 8 and 9. EBNA1 gene, showing the highest sensitivity, Kappa value and area under the ROC curve, makes it the most suitable single gene for the diagnosis of metastatic NPC in the fine-needle aspirate of neck masses. A slight compromise in specificity (97.1%) compared to EBNA2 (100%) should be acceptable. However, EBNA1 gene detection (88.3% sensitivity) is superior to FNAC where the diagnostic accuracy is 67.6% in this study (Table 18). It becomes very valuable in suggesting NPC as the origin of occult primaries and can guide the clinician in the diagnostic workup.

5.1.3 Paired gene detection in postnasal space biopsy

Taken in pairwise combinations (where two genes in a pair must both be detected to qualify as a 'positive'), the EBNA1-LMP1 combination seems to show the best sensitivity (91.4%) and specificity (100%) compared to the other two combinations which have lower sensitivities (88.6%) and equal or lower specificity (EBNA1-EBNA2 – 100%, EBNA2-LMP1 – 94.3%) – as shown in Table 10. . All three pairwise combinations have p values above 0.05 on McNemar's test (i.e. no significant difference from the gold standard of histopathological examination.) This indicates that pairwise-combinations are on par with the gold standard of HPE, supported by the Kappa statistic which shows that the EBNA1-LMP1 has an excellent inter-test agreement (0.91) followed by the EBNA1-EBNA2 combination (0.89) and EBNA2-LMP1 combination (0.83) – as shown in Table 10. The area-under-the-ROC-curve is best also for the EBNA1-LMP1 combination (0.96) but comparing the ROC curves, all three pairwise combinations show no significant difference from each other. ($p>0.05$) – as shown in Table 11 and 12. Therefore, the EBNA1-LMP1 combination is the most suitable pairwise combination of genes for the diagnosis of NPC in postnasal space biopsy. It is on par with HPE but not superior to the single gene detection of EBNA1. This can also be used as adjunct or alternative to diagnosis when it is not forthcoming from histopathological examination.

5.1.4 Paired gene detection in fine-needle aspirate of neck masses

The paired combination of EBNA2-LMP1 combination (where two genes in a pair must both be detected to qualify as a 'positive') shows the best sensitivity (82.4%) and specificity (100%) compared to the other two combinations which have equal or lower sensitivity and equal or lower specificity (Table 13). However, only the EBNA1-LMP1 combination shows a p value above 0.05 on McNemar's test (i.e. no significant difference from clinico-pathological criteria.) The Kappa statistic is best for the EBNA2-LMP1 with the highest inter-test agreement with histopathology (0.82) followed by the other two combinations (0.79). The area-under-the-ROC-curve is best also for the EBNA2-LMP1 combination (0.91) but comparing their ROC curves, all three pairwise combinations show no significant difference with each other ($p>0.05$) as shown in Table 14 and 15. Therefore, while the EBNA2-LMP1 combination is the best of the three pairwise-combinations, it is not on par to the gold standard of histopathology and not superior to the single gene detection of EBNA1.

5.1.5 Cut-off value analysis for total number of genes detected in postnasal space biopsy

Analysis of the cut-off point shows a reciprocal relationship where the sensitivity decreases and specificity increases with increasing cut-off point from >0/3 to >2/3. The >0/3 cut-off point gives the best sensitivity (97.1%) and lowest specificity (88.5%) while the >2/3 cut-off point has the highest specificity (100%) and lowest sensitivity (88.6%) – as shown in Table 16. All three cut-off points have p values above 0.05 on McNemar's test (i.e. no significant difference from HPE.) The >2/3 cut-off point has the highest value for Kappa statistic (0.89) and area-under-ROC-curve (0.94) – as shown in Table 17. However, comparing there area-under-ROC-curves, there is no significant difference between the three cut-off points with one another ($p>0.05$).

For a disease such as NPC, it is desirable to use a cut-off point which offers the highest sensitivity so as not to miss detecting a potentially curable disease (while a high specificity is desirable in a disease with grave prognosis.) Since there is no significant difference between the three cut-off points, it is arguably better to use the >0/3 or >1/3 cut-off points which offer better sensitivity (91.4-97.1%). It is on par with histopathological detection but not superior to single gene detection of EBNA1.

5.1.6 Cut-off value analysis for total number of genes detected in fine-needle aspirate of neck masses

Analysis of the cut-off points in fine-needle aspirates show a similar reciprocal relationship between sensitivity and specificity. The >0/3 cut-off point gives the best sensitivity (97.1%) and lowest specificity (94.1%) while the >2/3 cut-off point has the highest specificity (100%) and lowest sensitivity (79.4%) – as shown in Table 18. All three cut-off points have p values above 0.05 on McNemar's test (i.e. no significant difference from clinico-pathological criteria). The >0/3 cut-off point has the highest value for Kappa statistic (0.91) and also for the area-under-ROC-curve (0.93) – as shown in Table 18 and 19. However, comparing their area-under-the-ROC-curves, there seems to be no significant difference between the three cut-off points with one another ($p>0.05$).

Since >0/3 shows the best Kappa and area-under-ROC-curve values and offers the best sensitivity - which is ideal for a disease that is potentially curable - it is clearly the most suitable cut-off point in the diagnosis of metastatic NPC in fine-needle aspirate of neck masses. In fact, this is on par with HPE and superior to EBNA1 single-gene detection and EBNA2-LMP1 paired gene detection. This makes it ideal for the detection of metastatic NPC, especially in occult primaries. A summary table of the best detection methods for postnasal space biopsy and fine-needle aspirate of neck masses is presented in the table below.

Table 10 – Comparison between single gene, paired gene and cut-off point for postnasal space biopsy and fine-needle aspirate of neck masses.

	Sensitivity	Specificity	Inter-test agreement (Kappa)	Area under ROC curve	p value (McNemar's)
N series - Postnasal space biopsy					
Single gene EBNA1	**97.1**	**100**	**0.97**	**0.99**	**>0.05**
Paired gene EBNA1-LMP1	91.4	100	0.91	0.96	>0.05
Cut-off point > 0/3	97.1	88.6	0.77	0.93	>0.05
F series – Fine-needle aspirate of neck mass					
Single gene EBNA1	88.3	97.1	0.85	0.93	>0.05
Paired gene EBNA2-LMP1	82.4	100	0.82	0.91	<0.05
Cut-off point >0/3	**97.1**	**94.1**	**0.91**	**0.96**	**>0.05**

In summary, for postnasal space biopsy tissue, single-gene detection using EBNA1 is superior to paired-gene or cut-off point analysis in diagnosing NPC. One patient in this study was not known to be a case of NPC at the time of biopsy but EBV DNA (EBNA2 and LMP1) was detected in the postnasal space biopsy tissue. In follow up, this patient subsequently developed NPC within 1 year of the test. This may be explained by the presence of EBV DNA in the preinvasive state as described by Pathmanathan *et. al* (1995) or NPIN III (Nasopharyngeal intra-epithelial neoplasia stage III) as described by Huang and Lo (1999). In another case, biopsy failed to detect malignancy in an obvious tumour but subsequently confirmed it in a repeat biopsy 2 weeks later. EBV DNA was found in both specimens.

One must conclude that the detection of EBV genes is on par with HPE and that it can be an adjunct or alternative to diagnosis when the diagnosis is missed or when histopathological diagnosis is difficult for whatever reasons – e.g. non-representative biopsies, submucosal disease, technical difficulties in interpretation (Allen, 1999). It can also predict the development of NPC and therefore alert the clinician to perform serial biopsies and follow-up a patient very closely rather than dismissing him/her as normal with fatal results.

As for fine-needle aspirate of neck masses, a cut-off point of >0/3 is superior to either single-gene or paired-gene detection at the expense of a slight reduction in specificity compared to single-gene detection using EBNA1. In this study, a clinico-pathological criteria (i.e. clinically suspicious neck node in the presence of histopathologically confirmed NPC at the primary site) is taken as confirmatory of NPC. Barring open biopsy of a neck node, which worsens prognosis considerably (Cai *et al.*, 1983), a clinico-pathological criteria is the best means of diagnosing metastatic NPC. One must conclude that the detection of EBV genes in fine-needle aspirate tissue is on par with existing clinico-pathological criteria and by far superior to FNAC in detecting metastatic NPC. Therefore it is a valuable adjunct to guide the clinician in the diagnostic workup of occult primaries.

6 Conclusion

The final result of this study is presented according to their specific objectives, together with their validity data and statistical significance. These results have been obtained from an adequate sample size to achieve a power of study of 0.95.

6.1 Individual EBV gene detection for postnasal space biopsy

The EBNA1 gene is the best gene for detection of NPC in postnasal space biopsy tissue and is statistically superior to EBNA2 and LMP1 genes. It has a sensitivity of 97.1%, specificity of 100%, and inter-test agreement (Kappa) of 0.97 and is statistically on par with HPE as a diagnostic method.

6.2 Individual gene detection for fine-needle aspirate of neck masses

The EBNA1 gene is the best gene for detection of NPC in fine-needle aspirate tissue of neck masses and is statistically superior to EBNA2 and LMP1 genes. It has a sensitivity of 88.3%, specificity of 97.1%, an inter-test agreement (Kappa) of 0.85 and is statistically on par with present clinico-pathological criteria and superior to FNAC as a diagnostic method.

6.3 Paired gene detection in postnasal space biopsy

The EBNA1-LMP1 pair is the best combination of 2 genes for the detection of NPC in postnasal space biopsy tissue but it is not statistically superior to the other 2 pair-wise combinations. It has a sensitivity of 91.4%, specificity of 100%, an inter-test agreement (Kappa) of 0.91, and is statistically on par with HPE as a diagnostic method.

6.4 Paired gene detection in fine-needle aspirate of neck masses

The EBNA2-LMP1 combination shows the highest sensitivity (82.4%), specificity (100%), and inter-test agreement (Kappa) of 0.82 but it is not statistically equal to clinicopathological criteria. Therefore there is no valid pairwise combination for the detection of NPC in fine-needle aspirate of neck masses.

6.5 Cut-off value for total number of genes detected in postnasal space biopsy

A value of >0/3 is the best cut-off point to take in the detection of NPC in postnasal space biopsy tissue as it offers the highest sensitivity (97.1%) for detecting a potentially curable disease. At this cut-off point the specificity is 88.5% and inter-test agreement (Kappa) is 0.78, and it is statistically on par with HPE as a diagnostic method.

6.6 Cut-off value for total number of genes detected in fine-needle aspirate of neck masses

A value of >0/3 is the best cut-off point to take in the detection of NPC in fine-needle aspirate of neck masses as it offers the highest sensitivity (97.1%) for detecting a potentially curable disease. At this cut-off point the specificity is 94.1%; inter-test agreement (Kappa) is 0.91, statistically on par with present clinico-pathological criteria and superior to FNAC as a diagnostic method.

6.7 Presence of EBV DNA in histopathologically 'normal nasopharynx'

The detection of EBV DNA is predictive of nasopharyngeal carcinoma even when histopathology is unable to detect malignancy in postnasal space biopsy tissue.

6.8 EBV genes and histological type

There is no significant difference in the detection of EBV genes and histological type (according to WHO classification) in NPC.

In summary, EBV DNA detection in tissue obtained from postnasal biopsy and fine-needle aspirate of neck masses is a relatively cheap, reliable and accurate method of diagnosing NPC, and

1) It requires minimal amount of tissue and is rapid.
2) It is on par with histopathology in diagnosing NPC and on par with clinico-pathological criteria in detecting metastatic NPC.
3) It is superior to fine-needle cytology.
4) It can serve as an adjunct when histopathological diagnosis is missed and can predict the development of NPC.

It can also suggest NPC and guide the clinician in the diagnostic workup of occult primaries.

7 Bibliography

Akao, I., Sato, Y., Mukai, K., Sugano, S., Hoshikawa, T., Suzuki, T. & Tsutsumi, K. (1996). Localization of Epstein-Barr virus in lymph node metastasis with nasopharyngeal carcinoma. *Acta Otolaryngol Suppl,* **522,** 86-8.

Allen, P. W. S. P. I. v. H., C.A., Gibb, A.G. (Eds.), Nasopharyngeal Carcinoma 2nd ed. Chinese University Press. (1999). *Surgical Pathology.* In *Nasopharyngeal Carcinoma,* (Eds, van Hasselt, C. A. and Gibb, A. G.) Chinese University Press, pp. 69-103.

Cai, W. M., Zhang, H. X., Hu, Y. H. & Gu, X. Z. (1983). Influence of biopsy on the prognosis of nasopharyngeal carcinoma--a critical study of biopsy from the nasopharynx and cervical lymph node of 649 patients. *Int J Radiat Oncol Biol Phys,* **9,** 1439-44.

Chan, M. K. M., McGuire, L. & Lee, J. C. K. (1988). Fine Needle Aspiration Cytodiagnosis of Nasopharyngeal Carcinoma in Cervical Lymph Nodes - A Study of 40 Cases. *Acta Cytol,* **33,** 344-350.

sChang, A. R. & Chan, M. K. M. (1999). *Cytological diagnosis.* In *Nasopharyngeal Carcinoma 2nd ed,* (Eds, van Hasselt, A. and Gibb, A. G.) Chinese University Press, pp. 177-193.

Chang, A. R., Liang, X. M., Chan, A. T., Chan, M. K., Teo, P. M. & Johnson, P. J. (2001). The use of brush cytology and directed biopsies for the detection of nasopharyngeal carcinoma and precursor lesions. *Head Neck,* **23,** 637-45.

Cohen, J. I. & Kieff, E. (1991). An Epstein-Barr virus nuclear protein 2 domain essential for transformation is a direct transcriptional transactivator. *J. Virol,* **65,** 5880-5885.

Feinmesser, R., Feinmesser, M., Freeman, J. L., Noyek, A. M. & Livni, N. (1992a). Detection of occult nasopharyngeal primary tumours by means of in situ hybridization. *J Laryngol Otol,* **106,** 345-8.

Feinmesser, R., Miyazaki, I., Cheung, R., Freeman, J. L., Noyek, A. M. & Dosch, H. M. (1992b). Diagnosis of nasopharyngeal carcinoma by DNA amplification of tissue obtained by fine-needle aspiration. *N Engl J Med,* **326,** 17-21.

Haas, I., Hoffmann, T. K., Engers, R. & Ganzer, U. (2002). Diagnostic strategies in cervical carcinoma of an unknown primary (CUP). *Eur Arch Otorhinolaryngol,* **259,** 325-33.

Hao, S. P., Tsang, N. M. & Chang, K. P. (2003). Screening nasopharyngeal carcinoma by detection of the latent membrane protein 1 (LMP-1) gene with nasopharyngeal swabs. *Cancer,* **97,** 1909-13.

Ho, H. C., Ng, M. H., Kwan, H. C. & Chau, J. C. (1976). Epstein-Barr-virus-specific IgA and IgG serum antibodies in nasopharyngeal carcinoma. *Br J Cancer,* **34,** 655-60.

Huang, D. P. & Lo, K. (1999). *Aetiological factors and pathogenesis.* In *Nasopharyngeal Carcinoma 2nd ed.*, (Eds, van Hasselt, C. A. and Gibb, A. G.) Chinese University Press.

Kanegane, H., Nomura, K., Miyawaki, T. & Tosato, G. (2002). Biological aspects of Epstein-Barr virus (EBV)-infected lymphocytes in chronic active EBV infection and associated malignancies. *Crit Rev Oncol Hematol,* **44,** 239-49.

Kaur, A., Chew, C. T. & Lim-Tan, S. K. (1993). Fine needle aspiration of 123 head and neck masses--an initial experience. *Ann Acad Med Singapore,* **22,** 303-6.

Kwok, S. & Sninsky, J. J. (1999). *Application of PCR to the Detection of Human Infectious Diseases.* In *PCR Technology - Principles and Applications for DNA Amplification,* (Ed, Erlich, H. A.) Stockton Press.

Lee, W. Y., Hsiao, J. R., Jin, Y. T. & Tsai, S. T. (2000). Epstein-Barr virus detection in neck metastases by in-situ hybridization in fine-needle aspiration cytologic studies: an aid for differentiating the primary site. *Head Neck,* **22,** 336-40.

Macdonald, M. R., Freeman, J. L., Hui, M. F., Cheung, R. K., Warde, P., McIvor, N. P., Irish, J. & Dosch, H. M. (1995). Role of Epstein-Barr virus in fine-needle aspirates of metastatic neck nodes in the diagnosis of nasopharyngeal carcinoma. *Head Neck,* **17,** 487-93.

Ohshima, K., Kikuchi, M., Masuda, Y., Sumiyoshi, Y., Eguchi, F., Mohtai, H., Takeshita, M. & Kimura, N. (1991). Epstein-Barr viral genomes in carcinoma metastatic to lymph nodes. Association with nasopharyngeal carcinoma. *Acta Pathol Jpn,* **41,** 437-43.

Pacchioni, D., Negro, F., Valente, G. & Bussolati, G. (1994). Epstein-Barr virus detection by in situ hybridization in fine-needle aspiration biopsies. *Diagn Mol Pathol,* **3,** 100-4.

Pathmanathan, R., Prasad, U., Chandrika, G., Sadler, R., Flynn, K. & Raab-Traub, N. (1995). Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia. *Am J Pathol,* **146,** 1355-67.

Prasad, U. (1970) In *Proceedings of the Fifth Malaysian Singapore Congress of Medicine*(Ed, Prasad, U.) University of Malaya Press, Kuala Lumpur.

Prasad, U., Ablashi, D., Prathap, K., Yadav, M., Singaram, S. P., Singh, P. & Singh, J. (1983). *Problem of occult primary in nasopharyngeal carcinoma.* In *Nasopharyngeal Carcinoma: Current Concepts,* (Ed, Prasad, U.) University of Malaya Press, Kuala Lumpur, pp. 11-15.

Prasad, U. & Rampal, L. (1992). Descriptive epidemiology of nasopharyngeal carcinoma in Peninsular Malaysia. *Cancer Causes Control,* **3,** 179-82.

Raab-Traub, N., Flynn, K., Pearson, G., Huang, A., Levine, P., Lanier, A. & Pagano, J. (1987). The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. *Int J Cancer,* **39,** 25-9.

Shimakage, M., Horii, K., Tempaku, A., Kakudo, K., Shirasaka, T. & Sasagawa, T. (2002). Association of Epstein-Barr virus with oral cancers. *Hum Pathol,* **33,** 608-14.

Smith, S. S., Fowler, L. J., Hausenfluke, L., Cho, C. G., Eagan, P. A. & Gulley, M. L. (1995). Diagnosis of Epstein-Barr virus associated nasopharyngeal carcinoma using fine-needle aspiration biopsy and molecular diagnostics. *Diagn Cytopathol,* **13,** 155-9.

Tam, J. S. (1999). *Epstein-Barr Virus Serological Markers.* In *Nasopharyngeal Carcinoma 2nd ed.*, (Eds, van Hasselt, A. and Gibb, A. G.) Chinese University Press.

Tam, J. S. & Murray, H. G. (1990). Nasopharyngeal carcinoma and Epstein-Barr virus--associated serologic markers. *Ear Nose Throat J,* **69,** 261-7.

Tune, C. E., Liavaag, P. G., Freeman, J. L., van den Brekel, M. W., Shpitzer, T., Kerrebijn, J. D., Payne, D., Irish, J. C., Ng, R., Cheung, R. K. & Dosch, H. M. (1999). Nasopharyngeal brush biopsies and detection of nasopharyngeal cancer in a high-risk population. *J Natl Cancer Inst,* **91,** 796-800.

Van Hasselt, A. & Sing, F. L. (1999). *Clinical picture.* In *Nasopharyngeal Carcinoma,* Chinese University Press, pp. 105-110.

Walter, M. A., Menarguez-Palanca, J. & Peiper, S. C. (1992). Epstein-Barr virus detection in neck metastases by polymerase chain reaction. *Laryngoscope,* **102,** 481-5.

Wang, D., D., L. & E., K. (1985). An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell,* **43,** 831-840.

Wang, F., Gregory, C. D., Rowe, M., Rickinson, A. B., Wang, D., Birkenbach, M., Klkutanl, H., Kishimoto, T. & Kieff, E. (1987). Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. *Proc. Natl. Acad. Sci. USA,* **83,** 3452-3457.

Watkinson, J. C., M.N., G. & Wilson, J. A. (2000). *Tumors of the nasopharynx.* In *Stell & Maran's Head and Neck Surgery,* Butterworth-Heinemann, pp. 397-408.

Watson, J. D., Gilman, M., Witkowski, J. & Zoller, M. (1991). *The Polymerase Chain Reaction.* In *Recombinant DNA,* Scientific American Books.

Woo, J. (1999). *Clinical diagnosis.* In *Nasopharyngeal Carcinoma 2nd ed,* (Eds, van Hasselt, C. A. and Gibb, A. G.) Chinese University Press, pp. 111-126.

Yates, J. L., Warren, N. & Sngden, B. (1985). Stable replication of plasmid derived from Epstein-Barr virus in various mammalian cells. *Nature,* **313,** 812-815.

Pakistan Journal of Otolaryngology 2004; 20: 38-39 **Editorial**

Conventional Biopsy to EBV Amplification Kit in Quick Diagnosis of Nasopharyngeal Carcinoma

Shahid Hassan

Nasopharyngeal carcinoma (NPC) is the second most commonly occurring cancer amongst men after lung cancer in Malaysia.[1] The relative frequency in South East Asia and China is reported somewhere around 8% of all malignancies in specialized clinics. It is a leading cancer in Southern China and Hong Kong, the highest concentration being 18% in certain racial areas of this part of the world. On the average the annual incidence in Hong Kong is 23.3 per 100,000 population.[2]

Epstein Barr Virus (EBV) is associated with all types of NPC and the DNA of the virus is found in every cancer cell. EBV genomes consistently found in undifferentiated and well differentiated lesions have also been reported in pre-invasive state of NPC.[3] Current method of diagnosis is by biopsy of nasopharynx and histopathological examination. Delay in diagnosis may be attributed to some misleading symptoms of, neck mass (43-55%), rhinologic (20-30%) and otologic (10-13%).[4] Other factors include, late presentation occult primaries (13.3%), false negative biopsies (submucosal disease) and low accuracy for FNAC. Radiotherapy is the primary modality of treatment and now 100% cure has been claimed in T1 No lesions.

The conventional diagnostic method by nasopharyngeal biopsies and fine needle aspiration of neck lumps have high rate of false negatives. Serological test for EBV antibody titre is also less helpful due to low sensitivity and specificity. EBV DNA in tumor cells detected by polymerase chain reaction (PCR) has been shown to be more reliable and able to detect the cancer before it develops clinically. The author and his team in University Sciences Malaysia has successfully conducted a study to evaluate the validity and reliability of detecting EBV genes in fine needle aspirates and biopsy tissue in NPC by PCR. EBV products $EBNA_1$ and $EBNA_2$ and LMP_1 (generally implicated in oncogenesis) together with Beta-actin gene were analyzed with adequate sample in this study for the first time in Southeast Asia. This was a step forward towards the ultimate diagnosis of NPC which otherwise may lead to an advance disease with regional metastasis adversely affecting the prognosis due to delay in diagnosis. This and a few similar studies have given new hope towards the accurate diagnosis of NPC which has inherent pitfalls in detection by conventional methods based on histopathology.

This study has investigated the presence of EBV DNA in the tissue obtained from primary nasopharynx and the metastasis in the neck. A randomized case control study recently conducted in University Sciences Malaysia by Author et al with highest ever sample size, utilizing polymerase chain reaction method has shown the test statistically reliable with Kappa showing excellent inter-test agreement of 9.71 (HPE the gold standard) and likelihood ratio of >15 with single $EBNA_1$ gene detection. In two control subjects of the same study it was established that PCR analysis was helpful in predicting the lesion as NPC with an obvious lesion appearing later in 6 months. Similarly unknown primaries in few cases with positive tissues on the needle aspirates from occult neck metastasis, by this method were also proven to be NPC.

The outcome of this study suggests that EBV DNA detection is a highly sensitive and specific technique in diagnosing metastatic NPC and therefore may be used to guide the diagnostic workup of occult primaries. This will necessitate a careful repeat biopsy technique under direct vision from an anatomically occult area of nasopharynx. Further studies may be required to detect the rate of EBV DNA detection in other tumors like Hodgkin's, Burkit's and B-cell lymphoma and lympho-epithelial carcinoma of salivary gland from the Head and Neck region. However rarity makes it difficult to obtain tissue and investigate the conditions associated with EBV.

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Histopathology remains the gold standard for diagnosing NPC from the primary site, though it is not without limitations as discussed above. EBV DNA detection can help when HPE fails to detect the malignancy. In clinically doubtful cases, the presence of EBV DNA detection should raise the index of suspicions leading to a prompt repeat biopsy under general anaesthesia if necessary.

In non-suspicious cases EBV DNA may indicate pre-invasive stage to be followed up closely with repeat biopsy every 03 months. In occult primaries in Malaysian community, the presence of EBV DNA will hugely suggest NPC if lymphoma has been ruled out by cytology as NPC is very common here. Especially in Chinese and Malay races one is almost certain that occult primary with EBV DNA has originated from the nasopharynx.

Current Application - Developing a Diagnostic Kit: After the results obtained from this statistically significant study with a large sample size which has proven the presence of EBV DNA in NPC in Malaysia we can proceed to optimize this test. The very idea has helped us in creating a diagnostic kit that is cheap and simple to use as compared to a PCR based test which requires cold storage, reagent transportation and repeated freezing and thawing giving rise to erroneous results.

To achieve EBV amplification (EBV Amp) diagnostic kit, this test needed to be converted to multiplex form, i.e. all genes be amplified at once in a single tube and this is feasible as all 4 genes have discretely different product size and can easily be discerned on ethidium bromide stained electrophoresis gel. We have finally developed the EBV Amp Kit that contains thermostabilized PCR reagents in dry powder form in 0.5ml tube and can be easily transported without an ice box. The test has been proven to be 97% sensitive and 100% specific for EBV. The amplified product sizes are $EBNA_1$, $EBNA_2$, LMP_1 and Beta-human actin gene and an internal control DNA extraction from tissue performed in standard way can be added to thermostabilized PCR mix tube and operated in PCR. machine to be finally analyzed on UV trans-illuminator. The result obtained is interpreted in a specified manner. The test in rapid and a result can be obtained in 3 hours after adding 20 microlitre of extracted DNA into the test tube containing PCR mastermix, followed by polymerase chainreaction (PCR) and gel electrophoresis.

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This kit was also awarded the World Intellectual Property Organization as an overall best invention for year 2004 and the cost award, which covers the patenting cost.

References:

1. Parathp K. Prasad V. Ablash D.V. The pathology of nasopharyngeal carcinoma in Malaysia. In: nasopharyngeal carcinoma current concept. University of Malysia press, Kuala lumpur 1983.
2. Huang P. Dolly, Lo Kowk-Kai, Aetiological factors and pathogenesis. In: Nasopharyngeal carcinoma, 2nd edition. The Chinese University Press Hong Kong 1999.
3. De The G. Epidemiology of Epstein-Barr virus in man and associated disease in man. In: Herpes virus, vol. 1, Ed. Roimfzman, B. USA: Plenum press, 1982, 25-87.
4. Van Hassett C. Andrew, Leung Fai Sing. Clinical picture. In: Nasopharyngeal carcinoma, 2nd edition. The Chinese University Press Hong Kong 1999.

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INVITED EDITORIAL

A BREAKTHROUGH IN NPC DIAGNOSIS – FROM CONVENTIONAL BIOPSY TO EBV AMP KIT – *(The kit won the gold medal best invention for I-Tex 2004)*

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Nasopharyngeal carcinoma (NPC) is the second highest cancer amongst men in Malaysia (Lung Cancer being the first). Epstein Barr virus (EBV) is associated with all the types of NPC. In Peninsular Malaysia, the incidence of NPC is 365 cases per year or 5.4% of malignant tumors of nasopharynx (Prasad and Rampal, 1992); followed by lymphomas, adenocarcinomas and adenoid cystic carcinomas. It is potentially a curable disease if detected early in stage I or II; however in the advance stage, five years survival rate drastically falls from 100-80% (in stage I and II) to 58-38% (in stage III and IV). The diagnosis is sometimes delayed with increasing morbidity and mortality for non-representative biopsy, submucosal disease and occult primaries.

The conventional diagnostic method by nasopharyngeal biopsies and fine needle aspiration (FNA) of neck lumps have high rate of false negatives. EBV DNA in tumor cells detected by polymerase chain reaction (PCR) has been shown to be more reliable and able to detect the cancer early before the clinical manifestation. The author and his team in USM had successfully conducted a study under IRPA short-term grant to evaluate the validity and reliability of detecting EBV genes in FNA and biopsy tissue of NPC by PCR. Adequate sample of EBV products $EBNA_1$ and $EBNA_2$ and LMP_1 that are generally implicated in oncogenesis, together with Beta-actin were analyzed in this study conducted for the first time in Southeast Asia. This was a forward step towards the ultimate diagnosis of prospective NPC, otherwise it may lead to an advance disease with regional metastasis and adverse prognosis if diagnosed late. NPC is a unique cancer mostly presented with huge primary and regional metastasis, thus it demands an early diagnosis for

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with regional metastasis and adverse prognosis if diagnosed late. NPC is a unique cancer mostly presented with huge primary and regional metastasis, thus it demands an early diagnosis for better prognosis.

This and a few other similar studies have given new hope towards the accurate diagnosis of NPC which may have pitfalls in detection by conventional methods based on histopathology.

Application of this Study Test in Clinical Practice

This study investigated the presence of EBV DNA in metastatic neck of unknown primaries, i.e. NPC. The outcome of this study suggests that EBV DNA detection is a highly sensitive and specific technique in diagnosing metastatic NPC and therefore may be used to guide the diagnostic workup of occult primaries. This will necessitate a careful repeat biopsy technique under direct vision from an anatomically occult area of nasopharynx. Further studies may be required to detect the rate of EBV DNA detection in other tumors like Hodgkin's, Burkih's and B-cell lymphoma and lympho-epithelial carcinoma of salivary gland from the Head and Neck region which are rarely seen in Malaysia. However the scarcity of such cases makes it difficult to obtain tissue and investigate the conditions associated with EBV.

Histopathology remains the gold standard for diagnosis of NPC in the primary site, though it is not without limitations as discussed above. EBV DNA detection is especially useful when HPE fails to detect the malignancy. In clinically doubtful cases, the detection of EBV DNA should raise the index of suspicions leading to a prompt repeat of biopsy under GA if necessary. In non-suspicious cases EBV DNA may indicate pre-invasive stage to be followed up closely with repeat biopsy every 3 months. In Malaysia, the presence of EBV DNA in occult primaries will strongly suggest NPC if lymphoma has been ruled out by cytology, as NPC is very common here; especially in Chinese and Malays it is almost certain that occult primary with EBV DNA has originated from the nasopharynx.

Future Application – Developing a Diagnostic Kit

After obtaining the results from this statistically significant study with a large sample size which has proven the presence of EBV DNA in NPC, we can proceed to optimize this test. This will help us creating a diagnostic kit that is cheap and simple to use as compared to a PCR based test which requires cold storage, reagent transportation and repeated freezing and thawing that lead to erroneous results.

To create EBV amplification (EBV Amp) diagnostic kit, the test needs to be converted to multiplex form, i.e. all genes are simultaneously amplified in a single tube. This is feasible as all 4 genes have discretely different product size and can easily be discerned on ethidium bromide-stained electrophoresis gel. We have finally developed the EBV Amp Kit that contains thermo-stabilized PCR reagents in dry powder form in 0.5ml tube, which can be easily transported without cold chain. The test has been proven to be 97% sensitive and 100% specific for EBV. The amplified product sizes are EBNA1, EBNA2, LMP1 and Beta human actin gene, and an internal control. DNA extraction from tissue performed in standard way can be added to thermo-stabilized PCR mix tube and operated in PCR machine to be finally analyzed on UV trans-illuminator. The result obtained is interpreted in a specified manner. It is hoped that this single tube DNA test for rapid Epstein-Barr virus detection will prove a breakthrough in early diagnosis of NPC which is mandatory for a possible cure. After being patented (in process) EBV Amp kit will be available for general use in NPC practice at reasonable price together with detail of procedure, analysis of PCR product, interpretation criteria and precaution to be taken while performing the test.

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