EPSTEIN-BARR VIRUS SEROLOGY IN THE DIAGNOSIS OF NASOPHARYNGEAL CARCINOMA FROM SARAWAK

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ABSTRACT

The Epstein-Barr virus (EBV) is closely associated with nasopharyngeal carcinoma (NPC). The role(s) of EBV in NPC remains unclear but antibodies to two EBV proteins namely the viral capsid antigen (VCA) and early antigen (EA), are clinically useful diagnostic markers of NPC.

The NPC scenario in Malaysia has been based on studies done on West Malaysian patients but not much has been described for NPC in Sarawak, East Malaysia. In this study, 164 NPC sera from newly histologically confirmed NPC patients were collected from the Radiotherapy Unit of Kuching General Hospital, Sarawak prior to radiotherapy. One hundred and forty seven non-NPC controls that were sex, age and ethnic groups matched were also collected.

Among the NPC samples, the male to female ratio was 3.4 : 1 with mean age of 47.9 years. The ethnic group distribution among the 164 NPC cases was as follows: 69 cases of Ibans (42.1%), followed by Chinese (22.5%) and the Bidayuh (14.6%). The Ibans are 29.6% of the population while the Chinese make up 27.4%. The high incidence of Iban NPC patients is of interest since the origin of the Ibans is distinct from the Chinese who are known to have the highest incidence of NPC.

The immunofluorescence assay (IFA) for anti-EBV antibodies was used to titrate the serum IgA and IgG against VCA and EA. The present study found highly significant differences in the titres of all 4 markers (IgA/VCA, IgG/VCA, IgA/EA and IgG/EA) between NPC patients and the non-NPC controls. The geometric mean titres (GMT) for NPC sera were 34.8 for IgA/VCA, 412.4 (IgG/VCA), 14.9 (IgA/EA) and 76.0 (IgG/EA), compared to 2.3, 9.6, 2.0 and 3.1 in the non-NPC control sera. These anti-EBV titres were found independent of sex, ethnic groups and age.

For each serological marker, the sensitivity and specificity of the test determined the cut-off titre for positivity. A positive titre was ≥10, ≥160, ≥5 and ≥40 for IgA/VCA, IgG/VCA, IgA/EA and IgG/EA respectively. The GMT of the serological markers in NPC patients with elevated antibody levels was 55.6 for IgA/VCA, 576.5 for IgG/VCA, 29.0 for IgA/EA and 166.3 for IgG/EA. With a cut-off titre at ≥160, IgG/VCA was the most sensitive marker (89.0%) with a 98.0% specificity. For IgA/EA at a cut-off titre of ≥5, it was the most specific marker (100.0%) but the least sensitive (75.0%). The sensitivity and specificity of IgA/VCA was 83.6% and 97.3% at a cut-off titre at ≥10. IgG/EA had a sensitivity of 76.8% and specificity of 99.3% at a cut-off titre of ≥40.

A significant correlation was found between IgG/VCA (the most sensitive at ≥160) and the titres of the other 3 anti-EBV markers, indicating that if a patient has IgG/VCA antibodies, the other anti-EBV antibodies are likely elevated. One hundred and twelve (68.3%) of the 164 NPC patients were positive for all 4 markers, while 14 patients (8.5%) had no antibodies to any of the 4 EBV antigens. Eighteen (11.0%) NPC patients were positive for 3 markers, which included IgA/VCA, 10 (6.0%) NPC patients were positive for 2 markers, which also included 5 positive IgA/VCA samples and 10 (6.0%) NPC patients were positive for only 1 marker of which 2 were IgA/VCA positive. Based on the comparative sensitivity and specificity of the 4 anti-EBV antigens test, a combined use of IgG/VCA (≥160) and IgA/VCA (≥10) would improve the sensitivity of EBV serology in the diagnosis of NPC to 90.9%.
The role of EBV BHRF1 protein in NPC has been postulated to be through the prevention of apoptosis of EBV-infected cell during the early stages of NPC development, and antibodies to BHRF1 are detected in NPC patients. This protein might be a good marker for NPC diagnosis (Liu et al., 1998). For the determination of the BHRF1 protein linear epitope, 10-mer peptides were synthesised using the Multipin Peptide Synthesis kit. A total of 46 peptide fragments covering the entire 191 amino acids of BHRF1 were synthesised. The synthetic peptides of BHRF1 protein served as target antigens in ELISA and were tested for IgA specific antibodies in 5 NPC sera and 1 control serum. Five of the 46 synthetic peptides showed strong distinguishable IgA binding with NPC sera (peptides 6, 17, 26, 27 and 29). Peptide 17 (FTETWNRFIT) has the highest IgA specific binding and is a potential EBV antigen epitope candidate for NPC monitoring.
ABSTRAK

Virus Epstein-Barr (EBV) telah dikaitkan dengan karsinoma nasofarinks (NPC). Hubungan antara EBV dengan NPC masih tidak jelas tetapi antibodi terhadap dua protein EBV iaitu antigen kapsid virus (VCA) dan antigen awal (EA) memainkan peranan penting sebagai penanda dalam diagnosis NPC.

Senario NPC di Malaysia adalah berdasarkan kajian yang dijalankan di Semenanjung Malaysia tetapi tidak banyak data mengenai NPC di Sarawak, Malaysia Timur dilaporkan. Dalam kajian ini, 164 serum NPC diperolehi daripada pesakit sebelum radiasi dijalankan di Unit Radiotherapi, Hospital Besar Kuching, Sarawak. Pesakit-pesakit tersebut telah disahkan menghidap NPC secara histologi. Sebanyak 147 serum kawalan bukan NPC yang jantina, umur dan kaum berpanduan juga diperolehi.

Dikalangan sampel NPC, nisbah jumlah lelaki kepada perempuan adalah 3.4:1 dengan purata umur 47.9 tahun. Taburan kaum bagi 164 sampel tersebut adalah seperti berikut: kaum Iban dengan 69 kes (42.1%), diikuti kaum Cina (22.5%) dan Bidayuh (14.6%). Kaum Iban merangkumi 29.6% daripada populasi manakala kaum Cina merangkumi 27.4%. Bilangan kes pesakit NPC yang tinggi dikalangan kaum Iban adalah fenomena yang menarik memandangkan asal-usul kaum Iban berbeza daripada kaum Cina yang mana terkenal dengan bilangan kes NPC yang tinggi.

Teknik imunofluoresens (IFA) untuk antibodi anti-EBV digunakan untuk mengesakan IgA dan IgG terhadap VCA dan EA. Kajian ini menunjukkan perbezaan yang berterti dalam titer bagi kesemua 4 penanda (IgA/VCA, IgG/VGA, IgA/EA dan IgG/EA) diantara kumpulan NPC dan kumpulan kawalan. Titer purata geometrik (GMT) bagi kumpulan NPC adalah 34.8 untuk IgA/VCA, 412.4 (IgG/VGA), 14.9 (IgA/EA) dan 76.0 (IgG/EA) berbanding dengan 2.3, 9.6, 2.0 dan 3.1 masing-masing untuk kumpulan kawalan. Titer-titer anti-EBV yang diperolehi tidak bersandar kepada jantina, kaum dan umur pesakit NPC.

Titer ‘cut-off’ untuk kepositifan bagi setiap penanda ditentukan oleh kesensitifan dan kespesifik an ujian. Titer positif adalah \( \geq 10, \geq 160, \geq 5 \) dan \( \geq 40 \) bagi IgA/VCA, IgG/VGA, IgA/EA dan IgG/EA. GMT penanda serologi kumpulan NPC yang menunjukkan peningkatan takat antibodi adalah 55.6 untuk IgA/VCA, 576.5 untuk IgG/VCA, 29.0 untuk IgA/EA dan 166.3 untuk IgG/EA. Dengan titer ‘cut-off’ \( \geq 160 \), IgG/VCA merupakan penanda yang paling sensitif (89.0%) dengan kespesifik 98.0%. IgA/EA pada titer ‘cut-off’ \( \geq 5 \), merupakan penanda yang paling spesifik (100.0%) tetapi kurang sensitif (75.0%). Kesensitifan dan kespesifik IgA/VCA adalah 83.6% dan 97.3% pada titer ‘cut-off’ \( \geq 10 \). IgG/EA mempunyai kesensitif 76.8% dan kespesifik 99.3% pada titer ‘cut-off’ \( \geq 40 \).

Kolerasi diperolehi diantara IgG/VCA (penanda paling sensitif pada \( \geq 160 \)) dan titer terhadap 3 penanda anti-EBV yang lain. Ini bermakna jika sesearang pesakit mempunyai titer IgG/VCA, antibodi anti-EBV bagi penanda yang lain juga besar kemungkinan adalah tinggi. Seratus dua belas (68.3%) daripada 164 pesakit NPC adalah positif terhadap keempat-empat penanda manakala 14 pesakit (8.5%) tidak mempunyai antibodi terhadap mana-mana 4 penanda tersebut. Lapan belas orang (11.0%) pesakit NPC adalah positif terhadap 3 penanda, termasuk IgA/VCA, 10 orang (6.0%) pesakit adalah positif terhadap 2 penanda, termasuk 5 sampel positif terhadap IgA/VCA dan 10 orang (6.0%) pesakit adalah positif terhadap 1 penanda sahaja,
termasuk 2 sampel positif terhadap IgA/VCA. Berdasarkan perbandingan kesensitifan
dan kespesifikan terhadap ujian bagi 4 antigen anti-EBV, penggunaan kedua-dua
penanda IgG/VCA (pada ≥160) dan IgA/VCA (pada ≥10) dapat mempertingkatkan
kesensitifan serologi EBV terhadap diagnosis NPC kepada 90.9%.

Protein EBV BHRF1 dalam NPC dicadangkan berperanan mencegah apotosis
dalam sel yang dijangkiti EBV semasa fasa awal perkembangan NPC. Antibodi
terhadap BHRF1 dapat dikesan dalam pesakit NPC. Protein ini mungkin berguna
sebagai penanda untuk diagnosis NPC (Liu et al., 1998). Untuk penentuan epitop
linear protein BHRF1, peptida 10-mer disintesis dengan menggunakan kit ‘Multipin
Peptide Synthesis’. Sebanyak 46 set peptida yang merangkumi kesemua 191 asid
amino bagi BHRF1 telah disintesis. Peptida sintetik BHRF1 digunakan sebagai
antigen dalam ELISA untuk mengesan kehadiran antibodi spesifik IgA dalam 5 serum
NPC dan 1 serum kontrol. Lima daripada 46 peptida sintetik (Peptida 6, 17, 26, 27
dan 29) menunjukkan reaktiviti yang tinggi terhadap antibodi IgA. Peptida 17
(FTETWNRFIT) telah mempamerkan ikatan antibodi spesifik IgA tertinggi dan oleh
itu berpotensi untuk digunakan sebagai epitop antigen EBV untuk tujuan pemantauan
NPC.
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LIST OF ABBREVIATIONS

< : Less than
> : Greater than
°C : Degree Celsius
µg : Microgram
µl : Microlitre
µm : Micrometer
BHRF : BamH1 fragment right reading frame
BL : Burkitt’s lymphoma
bp : Base pairs
DIC : Diisopropylcarbodiimide
DMF : N,N-dimethylformamide
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleotide acid
EA : Early antigen
EA-D : Early antigen, diffuse component
EA-R : Early antigen, restricted component
EBER : Epstein-Barr virus encoded small nonpolyadenylated RNA
EBNA : Epstein-Barr virus nuclear antigen
EBV : Epstein-Barr virus
ELISA : Enzyme-linked immunosorbent assay
FITC : Fluorescein isothiocyanate
F-moc : Fluorenlymethoxy carbonyl
g : Gram
GMT : Geometric mean titre
HHV4 : Human Herpes Virus 4
HLA : Human leukocyte antigen
HOBr : 1-hydroxybenzotriazole
IFA : Immunofluorescence Assay
IMR : Institute of Medical Research
Ig : Immunoglobulin
IM : Infectious mononucleosis
IR : Internal repeat
IU : International unit
kD : Kilodalton
LCLs : Lymphoblastoid cell lines
LMP : Latent membrane protein
LP : Leader protein
M : Molar
MA : Membrane antigen
mg : Miligram
ml : Millilitre
mRNA : Messenger ribonucleic acid
NBA : Sodium butyrate
NKC : Non-keratinizing carcinoma
NPC : Nasopharyngeal carcinoma
OPD : o-phenylenediamine
PBS : Phosphate buffered saline
RNA : Ribonucleotide acid
rpm : Revolutions per minute
RPMI : Rosewell Park Memorial Institute Medium
RTU : Radiotherapy Unit
SSC : Squamous cell carcinoma
TFA : Trifluoroacetic acid
TPA : 12-o-tetradecanoyl phorbol 13-acetate
TR : Terminal repeats
UC : Undifferentiated (anaplastic) carcinoma
UCNT : Undifferentiated carcinoma of nasopharyngeal type
UL : Unique long sequence
US : Unique short sequence
VCA : Viral capsid antigen
WHO : World Health Organisation
ZEBRA : BamHI Z Epstein-Barr virus replication activator