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Epstein-Barr Virus Serology in the Detection and Screening of Nasopharyngeal Carcinoma

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1. Introduction

Nasopharyngeal carcinoma (NPC) is a common cancer among Southern Chinese with a male dominance of about 3:1. The age-adjusted incidence for both sexes is less than one per 100, 000 population worldwide. The reported incidence of NPC among men and women in Hong Kong is 20–30 per 100, 000 and 15–20 per 100, 000.(Wei and Sham, 2005) The reported incidence of NPC among men and women in Taiwan is 8.3 per 100 000 and 2.8 per 100 000, respectively.(Bureau of health promotion, Taiwan, 2010) It mainly afflicts people in mid-life. There is now compelling evidences to suggest that Epstein-Barr virus (EBV) is associated with the development of NPC and is most likely to be involved in the multi-step and multi-factorial carcinogenesis of NPC. In this chapter, the role of EBV in pathogenesis of NPC is reviewed briefly, and principle applications of EBV antibodies and circulating EBV DNA as markers of NPC are outlined. Based on current knowledge of EBV antibody responses by NPC and taking available testing technologies into account, serologic screening strategy to facilitate efficient early detection of NPC is formulated.

2. EBV related pathogenesis of NPC

The pathogenesis of NPC includes multi-stepped process that leads to the development of NPC **(Fig. 1.).** EBV infection alone cannot drive normal cells towards carcinoma development. It is thought that loss of heterozygosity (LOH on chromosome 3p and 9p, which are the location of some tumor suppressor genes), possibly as a result of inherited traits (Chinese ethnicity) as well as exposure to dietary factors (salted fish) and other environmental cofactors (Formaldehyde), is an early stage event in the pathogenesis of this disease. EBV is infected within these low-grade pre-invasive lesions, subsequent to further genetic and epigenetic alterations.

EBV was first suspected to be linked with NPC on the basis of the serological observations by Old and colleagues (Old et al., 1966) in 1966. This link was formally demonstrated later by in situ hybridization of the viral DNA in the nuclei of epithelial cells (zur Hausen et al., 1970). The full length EBV genome is contained in all malignant epithelial cells, but not in most infiltrating lymphocytes. The association with EBV is constant, regardless of the

patient's geographical origin and is observed in World Health Organization (WHO) types II and III. However, the association of NPC type I with EBV has long been a matter of controversy. It is now clear that WHO type I tumors are frequently associated with EBV in endemic regions, but not in non-endemic regions, where they often result from tobacco and alcohol abuse (Nicholls et al., 1997). Types II and III may be accompanied by an inflammatory infiltrate of lymphocytes, plasma cells, and eosinophils.
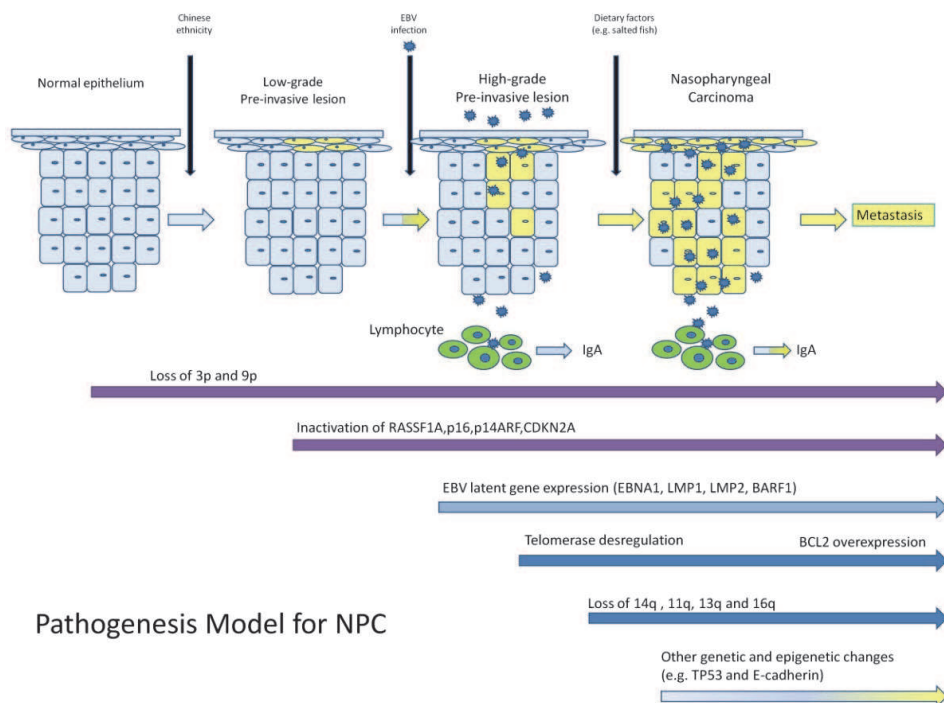

Fig. 1. EBV in the pathogenesis model for nasopharyngeal carcinoma

More than 95% adults in all ethnic groups across the world are healthy carriers of EBV (Ooka et al., 1991). This means that NPC oncogenesis is not simply a consequence of EBV infection. It probably results from a form of viral reactivation in combination with other events, such as cellular genetic lesions due to environmental carcinogens and/or some form of immune defects. EBV-encoded RNA signal (EBER) has been shown, by in-situ hybridization, to be present in nearly all tumor cells, whereas EBV-encoded RNA is absent from the adjacent normal tissue, except perhaps for a few scattered lymphoid cells. Premalignant lesions of the nasopharyngeal epithelium have also been shown to harbor EBV, which suggests that the infection occurs in the early phases of carcinogenesis (Gulley, 2001). Consistent with this hypothesis, is the fact that NPC generally occur several years after EBV primary infection. The expression of EBV latent genes provides growth and survival advantages to these infected cells, ultimately leading to the development of NPC. Further genetic and epigenetic alterations post-NPC development can occur, which may result in a more metastatic disease. Because of it takes years for premalignant lesion after

EBV infection to NPC formation(Choi et al., 2011; Ji et al., 2007; Ng et al., 2010; Ng et al., 2005), the long period of pre-clinical detectable phase (PCDP) offer the opportunity for screening and early diagnosis of NPC.

3. Applications of EBV antibodies and EBV DNA as markers of NPC in population

3.1 Plasma EBV protein in the diagnosis of NPC

Serological studies have shown that the clinical onset of NPC is preceded by the appearance of a high titer of various EBV antibodies such as viral capsid antigens (VCA) IgA(Li et al., 2010; Ng et al., 2010; O et al., 2007), anti-EBV DNase(Chien et al., 2001) and combined EBV EA(early antigen) + EBNA-1 (Nuclear antigen 1) IgA test(Chang et al., 2008)(**Table 1.**).

Study type	Author	Marker	Results of the utility
Case-control study	O *et al.* (O et al., 2007)	VCA IgA (ELISA)	Sensitivity 90.6% Specificity 93.5%
	Ng *et al.* (2010) (Ng et al., 2010)	VCA IgA (ELISA and IF)	Sensitivity 83.3% Specificity 87.0%
	Chang *et al.* (2008) (Chang et al., 2008)	EA+EBNA1 IgA (ELISA)	Sensitivity 94.2% Specificity 82.6%
Meta-analysis	Li *et al.* (2010) (Li et al., 2010)	VCA IgA (ELISA and IF)	Sensitivity 92% Specificity 98%
Cohort study	Chien *et al.* (2001) (Chien et al., 2001)	VCA IgA(IF) and anti-DNase (enzyme neutralization assay)	Rate ratio VCA IgA 22.0 (7.3–66.9) anti-DNase 3.5 (1.4–8.7)
	Yu *at al.* (2011) (Yu et al., 2011).	EBNA1 IgA (ELISA)	Rate ratio 4.7 (1.4–16)

IF: immunofluorescent assay; ELISA: enzyme-linked immunosorbent assay

Table 1. Reported plasma EBV antibody in NPC diagnosis and risk assessment of NPC

For NPC serodiagnosis, cell-based indirect immunofluorescent assay (IF) methods are widely considered the gold standard (Paramita et al., 2009). IF involves the separate analysis of antibody responses to VCA, EA, and EBNA, and requiring different cell lines for specific analysis. However, this method shows considerable variation among laboratories and is time-consuming, subjective, and not suitable for large-scale automatic handling. Enzyme-linked immunosorbent assay (ELISA) techniques are increasingly used recently and have shown a better sensitivity and specificity compared to IFA and which are suitable for large-scale application. Stage is one of the most important prognostic predictors of NPC. NPC in its early stages are highly curable with radiotherapy. Screening may change the distribution of stage and prognosis. In a cohort study undertaken in Wuzhou (Guangxi province, China) in the early 1980s (Zeng et al., 1985), total 1136 individuals identified as positive for Ig A

against VCA received regular clinical examinations of the nasopharynx and neck for 4 years. During this follow-up period, 35 cases of NPC were detected, most of which (92%) were diagnosed early at either stage I or stage II. The annual detection rate of NPC for this group was 31·7 times higher than for the population as a whole. Distribution of different stages of screen-detected and symptomatic NPC in Hong Kong (Ng et al., 2010) also revealed early diagnosis of NPC in screen-detected patients, comparing to symptomatic NPC (**Table 2.**).

	Stage I	Stage II	Stage III	Stage IV
Screen-detected	41.2%	17.6%	35.3%	5.9%
Clinically detected	0.7%	23.2%	36.3%	39.5%

Table 2. The comparison of the stage distribution between screen-detected and clinically detected based on published data on NPC screening during 1979–1992 (Ng et al., 2010).

Li conducted a systematic review of studies in Chinese on the accuracy of VCA-IgA concentrations in the diagnosis of NPC using random effects models (Li et al., 2010). Twenty studies met the inclusion criteria for the meta-analysis. The summary estimates (**Fig. 2.**) for VCA-IgA in the diagnosis of NPC were: sensitivity 0.92 (95% confidence interval (CI): 0.89–0.95), specificity 0.98 (95% CI: 0.95–0.99), positive likelihood ratio 38.5 (95% CI: 19.0–78.0), negative likelihood ratio 0.08 (95% CI: 0.05–0.12) and diagnostic odds ratio 487 (95% CI: 224–1059). The area under the summary receiver operating characteristic curves was 0.98(95%
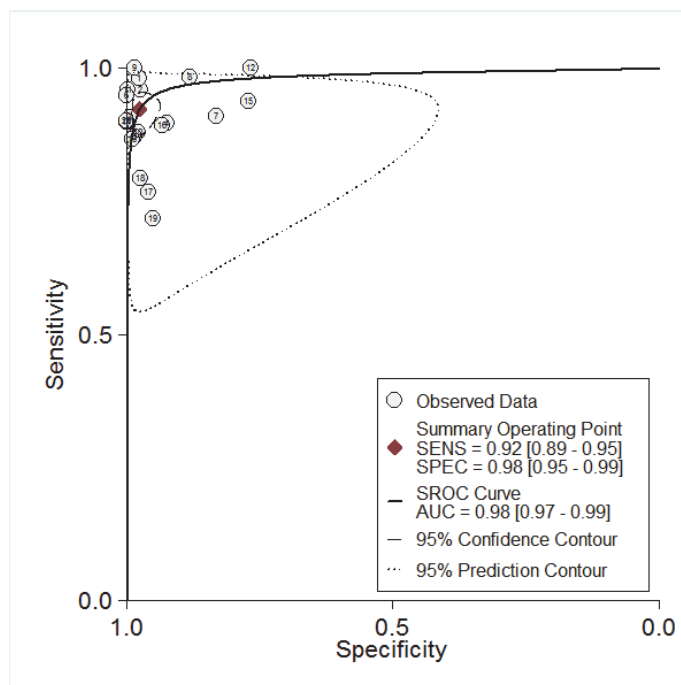

Fig. 2. Summary receiver operating characteristic curves for VCA-IgA in the diagnosis of NPC. Each solid circle represents each study in the meta-analysis.

CI: 0.97–0.99, **Fig. 3.**). There are limitations of this meta-analysis: (1) All of the included studies were Chinese articles, which would lead to language bias. (2) Significant heterogeneity exists in this metaanalysis, even with random effect model, pooling is not a proper method.

StudyId	SENSITIVITY (95% CI)	StudyId	SPECIFICITY (95% CI)
Li, 2007	0.90 [0.88 - 0.92]	Li, 2007	1.00 [0.98 - 1.00]
Li, 2007	0.72 [0.58 - 0.83]	Li, 2007	0.95 [0.83 - 0.99]
Huang, 2006	0.79 [0.73 - 0.85]	Huang, 2006	0.98 [0.91 - 1.00]
Huang, 2005	0.77 [0.66 - 0.85]	Huang, 2005	0.96 [0.86 - 1.00]
Huang, 2003	0.89 [0.81 - 0.95]	Huang, 2003	0.93 [0.84 - 0.98]
Fang, 2003	0.94 [0.88 - 0.97]	Fang, 2003	0.77 [0.74 - 0.80]
Wang, 2000	0.90 [0.80 - 0.96]	Wang, 2000	1.00 [0.88 - 1.00]
Zhou, 2000	0.87 [0.75 - 0.94]	Zhou, 2000	0.99 [0.97 - 1.00]
Huang, 1998	1.00 [0.97 - 1.00]	Huang, 1998	0.77 [0.74 - 0.79]
Xiao, 1995	0.96 [0.95 - 0.97]	Xiao, 1995	1.00 [1.00 - 1.00]
Zhou, 1995	0.88 [0.76 - 0.95]	Zhou, 1995	0.98 [0.89 - 1.00]
Tian, 1990	1.00 [0.69 - 1.00]	Tian, 1990	0.98 [0.97 - 0.99]
Huang, 1988	0.98 [0.91 - 1.00]	Huang, 1988	0.88 [0.76 - 0.96]
Liu, 1987	0.91 [0.82 - 0.96]	Liu, 1987	0.83 [0.73 - 0.90]
He, 1986	0.95 [0.89 - 0.98]	He, 1986	1.00 [0.93 - 1.00]
Kuang, 1985	0.87 [0.84 - 0.90]	Kuang, 1985	0.99 [0.96 - 1.00]
Gao, 1984	0.90 [0.81 - 0.95]	Gao, 1984	0.93 [0.76 - 0.99]
Li, 1983	0.91 [0.89 - 0.93]	Li, 1983	0.99 [0.98 - 1.00]
Xie, 1983	0.96 [0.90 - 0.99]	Xie, 1983	0.97 [0.93 - 0.99]
Tang, 1981	0.98 [0.95 - 1.00]	Tang, 1981	0.98 [0.94 - 0.99]
COMBINED	0.92[0.89 - 0.95]	COMBINED	0.98[0.95 - 0.99]
	Q =148.35, df = 19.00, p = 0.00		Q =1746.15, df = 19.00, p = 0.00
	I2 = 87.19 [82.56 - 91.82]		I2 = 98.91 [98.73 - 99.09]

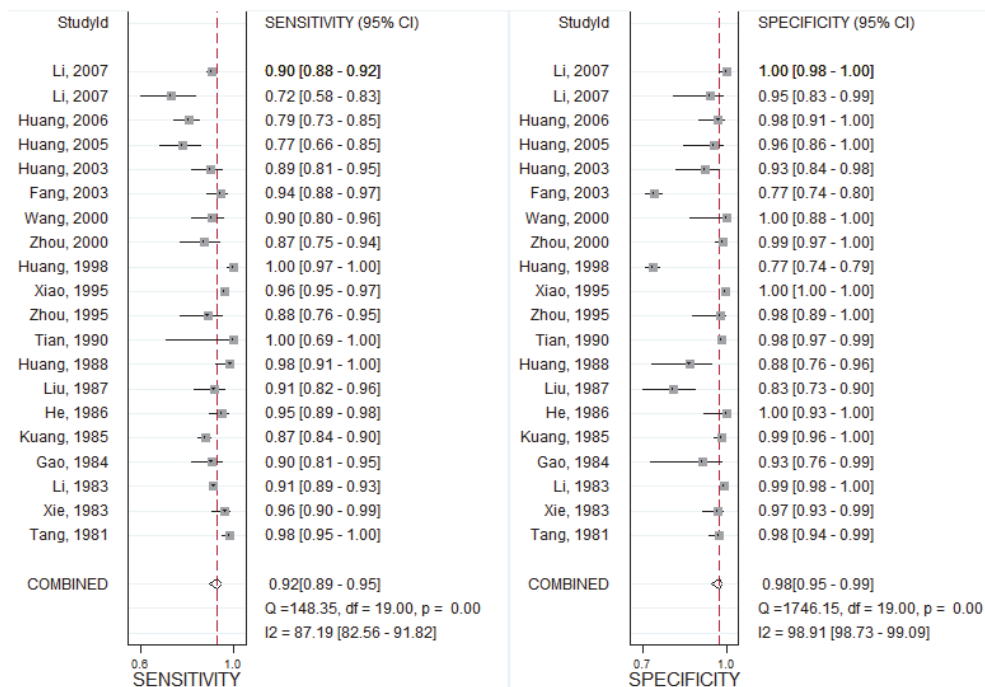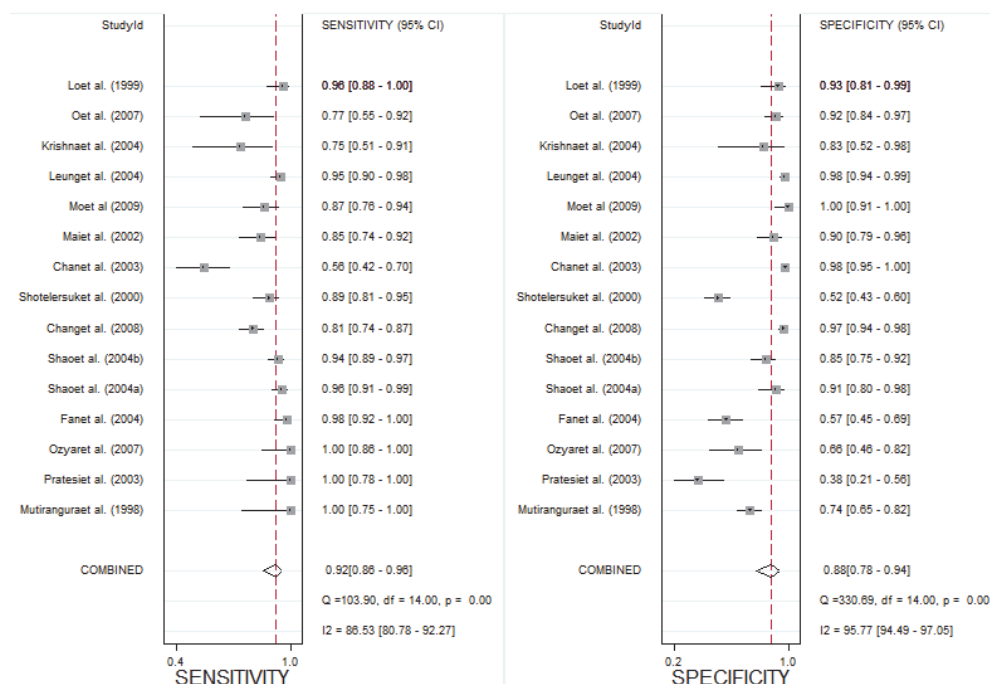SENSITIVITY (0.6 – 1.0)   SPECIFICITY (0.7 – 1.0)

Fig. 3. Forest plot of estimates of sensitivity and specificity for VCA-IgA in the diagnosis of NPC. The point estimates of sensitivity from each study are shown as solid squares. Error bars are 95% *CI*.

Sustained elevation of EBV antibody had a high possibility of NPC formation. Ji monitored serologically and clinically 39 cases for different periods of up to 15 years before NPC was diagnosed, and assessed the preclinical serologic status of another 68 cases(Ji et al., 2007). The results identify a serologic window preceding diagnosis when antibody levels are raised and sustained. This window can persist for as long as 10 years, with a mean duration estimated to as 37 months.

3.2 Circulating EBV DNA in the diagnosis of NPC

The presence of circulating DNA was first reported by Mandel and Metais in 1948 (Chan and Lo, 2002). They demonstrated that extracellular DNA and RNA could be detected from the blood of healthy as well as sick individuals. The blood plasma EBV DNA load was shown to be proportionately related to the presence of malignant disease (Tan et al., 2006). While the EBV copy number in untreated NPC patients had a median of 2,043 copies/ml, viral load in plasma of healthy controls was significantly lower (median of 0 copy/ml). The

demonstration of EBV DNA in the plasma/serum of patients suffering from NPC has provided us with a new tool for NPC detection and monitoring.

Liu reported a systematic review of 15 studies in English on the accuracy of EBV-DNA in the diagnosis of NPC (Liu et al., 2011). NPC could be diagnosed by detecting plasma or serum EBV DNA. Pooling using random effects models showed that EBV DNA detection was also highly sensitive and specific for cancer detection, and can possibly help the clinician to diagnose NPC. The summary estimates (**Fig. 4.**) for DNA in the diagnosis of NPC were: sensitivity 0.92 (95% confidence interval (CI): 0.82–0.96), specificity 0.88 (95% CI: 0.78–0.94), positive likelihood ratio 7.7 (95% CI: 4.1–14.6), negative likelihood ratio 0.09 (95% CI: 0.05–0.15) and diagnostic odds ratio 89 (95% CI: 44–181). The area under the summary receiver operating characteristic curves was 0.96(95% CI: 0.94–0.97, **Fig. 5.**).

Fig. 4. Forest plot of estimates of sensitivity and specificity for EBV DNA in the diagnosis of NPC. The point estimates of sensitivity from each study are shown as solid squares. Error bars are 95% CI.

The presentation with "occult primary" of NPC is a diagnostic challenge for the clinician. Detection of EBV DNA by PCR in metastatic neck nodes has a good diagnostic rate (97.1%) (Yap et al., 2007). PCR is an ideal tool for suggesting occult primary NPC and guiding the diagnostic workup, facilitating earlier diagnosis and reducing morbidity and mortality. It is, therefore, expected that this promising molecular tumor marker would soon be incorporated into routine clinical use.
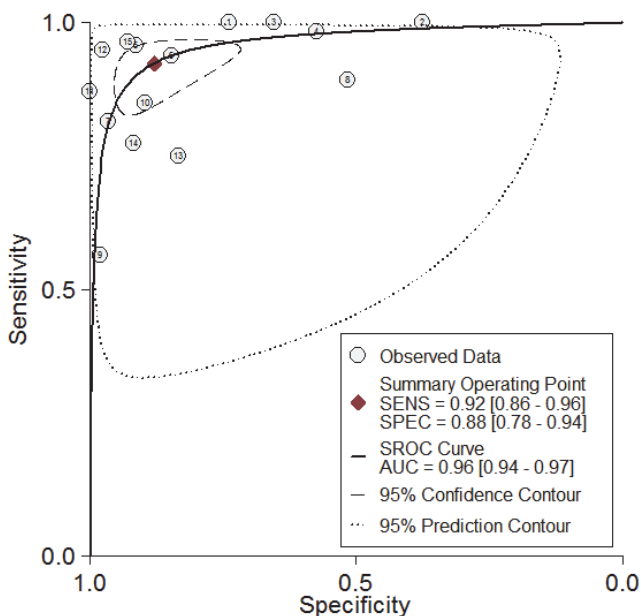
Fig. 5. Summary receiver operating characteristic curves for VCA-IgA in the diagnosis of NPC. Each solid circle represents each study in the meta-analysis.

3.3 EBV DNA in the monitoring of NPC

With the demonstration of the presence of extracellular EBV DNA in the circulation of NPC patients and its disappearance following radiotherapy, the levels of plasma/serum EBV DNA in patients with NPC recurrence were much higher than the levels of those who remained in continuous clinical remission. A significant decrease in EBV load was observed in patients who had undergone radiotherapy while a high viral load indicated in patients correlated to tumor relapse and presence of distant metastasis upon clinical investigation(Lo et al., 1999; Tan et al., 2006). The median EBV DNA concentration in the relapsed patients was 32, 350 copies/ml, whereas that in patients in remission was 0 copy/ml (Lo et al., 1999). Plasma EBV DNA is superior to serum EBV VCA antibodies in prognostic predictions and monitoring for NPC (Twu et al., 2007). Relapsed patients had significantly higher pretreatment EBV DNA concentration than patients without relapse (p<0.05). No associations of VCA-IgA (p =0.97) or VCA-IgG (p=0.61) were observed between patients with and without relapse (Twu et al., 2007).

3.4 Comparison of DNA and serology in the diagnosis and follow-up of NPC

Plasma EBV DNA detection has a similar sensitive and specific as the serum IgA antibody titer for the diagnosis of patients with NPC. In terms of real costs, however, the IgA test costs approximately $20, whereas the DNA test ranges from $100 to $200 (5-10 times more)(O et al., 2007). For screening purposes, the serologic assays may be made first, so that more patients can be evaluated. If a patient is assessed to be at high risk, then EBV DNA

PCR can be performed in a series fashion (O et al., 2007). If this work-up is positive; then the patient should be referred to a specialist for further work-up including fiberoptic nasopharyngoscopy or MRI examination. The economic cost of providing this screening is reduce by prescreening to exclude the low-risk patients and performing series testing on the at-risk patients.

4. Applications of EBV antibodies and EBV DNA as markers of NPC among individual from high risk NPC families

4.1 Screening in high risk group with positive family history

The incidence of NPC is relative low through most of the world (less 1/100,000). However, familial clustering of NPC has been widely documented in Chinese population (Chen and Huang, 1997) and individual with family history are at a high risk of developing the disease. Both family history and anti-EBV seropositivity are determinants in subsequent NPC development. Based on mass screening with positive family history conducted in Taiwan and Hong-Kong (Choi et al., 2011; Hsu et al., 2011; Ng et al., 2010; Yu et al., 2011), subjected with positive family history will increase risk of subsequent NPC development. Screening this high risk group significantly shifted to earlier stage (Ng et al., 2010), which mean it is a screening modality to be worth.

The etiology of NPC is not completely understood now; approaches to primary prevention of NPC remain inconclusive. This situation highlights the need of secondary prevention for early detection, diagnosis, and treatment of NPC. The effectiveness of a screening program rests on several key factors: "the importance of the disease; a well-defined population at risk; the availability of noninvasive, low-cost screening tests that can detect disease at an early stage; and effective treatments resulting in long term survival."(Choi et al., 2011) Screening for NPC for positive family history with EBV serology meets all of these criteria.

Chen first reported an evaluation of screening for NPC using Markov chain models (Chen et al., 1999). Average duration of the PCDP is estimated as 3.1 years was estimated; therefore it is possible for massive screening in NPC. The stage distribution for EBV-IgA antibodies is higher (68.7%) than in the non-screened (25%) group for stage I and II. Based on these findings, they suggest design a randomized trial in a high incidence area such as Hong Kong.

In another cohort study from Taiwan (Hsu et al., 2011) comparing different covariates, the authors compared the long-term risk NPC of male participants in a NPC cohort after adjusting for anti- EBV seromarkers and cigarette smoking. The adjusted hazard ratio was 6.8 (95% CI: 2.3, 20.1) for the multiplex family cohort compared with the community cohort. In the evaluation of anti-EBV VCA IgA and anti-EBV DNase, the adjusted hazard ratios were 2.8 (95% CI: 1.3, 6.0) and 15.1 (95% CI: 4.2, 54.1) for those positive for 1 EBV seromarker and positive for both seromarkers, respectively, compared with those negative for both EBV seromarkers. The adjusted hazard ratio was 31.0 (95% CI: 9.7, 98.7) for participants who reported a family history of NPC and who were anti-EBV-seropositive compared with individuals without such a history who were anti-EBV-seronegative. These findings suggest that both family history of NPC and anti-EBV seropositivity are important determinants of

subsequent NPC development. Screening for high risk multiplex family may be more cost-effective.

Screening in Hong-Kong with positive NPC family history was conducted since 1994 (Lee et al., 2005; Ng et al., 2010; Ng et al., 2005). Participants in this screening program for NPC were all first degree relatives of patients with NPC and they were all 18 years of age or older. Participants were offered annual assessment serological test of EBV and endoscopic examination of the nasopharynx. Between 1994 and 2005, total 1,199 asymptomatic family members of NPC patients were recruited and reported (Ng et al., 2010). Eighteen participants of the screening program developed NPC; 16 of them were detected in the screening. Stage distributions and survival outcomes of the 17 cases were compared with that of 1,185 consecutive symptomatic patients diagnosed in the same period through general referral. It was found that the screening program resulted in early detection of cancer (Table 2), with 59% presenting at early stage (stage I: 41%, stage II: 18%) compared to 24% (stage I: 1%, stage II: 23%) of symptomatic cancers (P=0.001), and a significant improvement in disease-free survival (P = 0.04). The cancer specific survival and overall survival rate at 5-year are also higher (92 vs. 77% and 92 vs. 70%, respectively).

4.2 Screening strategies of NPC among individual from high risk NPC families

The efficacy of any screening strategy should be evaluated before putting it into practice. Based on Markov chain models, Choi simulated and compared the outcomes of 4 screening strategies over a period of 12 years: (A) Annual screening, (B) biennial screening, (C) triennial screening, and (D) triennial screening for participants tested EBV negative and annual screening once the participants are tested EBV positive (Choi et al., 2011) . The result is summarized at **Table 3**, strategy A (screening annually) yields the maximum disease pick-up rate, but strategy D (triennial screening for participants tested EBV negative and annual screening once the participants are tested EBV positive) offered the highest efficacy for NPC screening of family members of NPC patients.

	Strategy A	Strategy B	Strategy C	Strategy D	No screening
Total screens	77,652	41,837	29,898	44,618	-
Positive EBV test	14,962	8,071	5,772	11,413	-
Screen detected case	47	42	38	47	-
Disease pick up rate	88.2%	78.6%	70.8%	87.4%	-
Reduction in disease pick up rate*	-	9.6%	17.3%	0.8%	-
Screen missed cases	7	12	16	7	-
5-year overall survival	80.1%	78.8%	77.7%	80.0%	67.9%

* Relative to strategy A
Strategy A. Annual screening
Strategy B. Biennial screening
Strategy C. Triennial screening
Strategy D. Triennial screening for participants tested EBV negative and annual screening once the participants are tested EBV positive

Table 3. Simulated screening result with the four strategies based on an imaginary population of 6,000 participants follow up for 12 years with family history of NPC (Choi et al., 2011).

Various EBV screening seromarkers were ever been reported for massive screening in high risk group with positive family NPC history (**Table 4.**) (Ng et al., 2010; Yu et al., 2011). The positive predicative value (PPV) of EBV seromarkers was below 10% and the negative predicative value (NPV) was higher than 99%. Because the average duration of the preclinical screen-detectable phase is estimated as 3.1 years, annual check-up once the participants are tested EBV positive is necessary. For the high NPV, triennial screening is reasonable for NPC family history with negative EBV seromarkers.

Author	Journal	Modality	Sensitivity/ Specificity	Positive%	NPV	PPV
Wai *el al.* (Ng et al., 2010) (Hong-Kong)	Fam Cancer (2010)	Anti-EBV VCA IgA	83.3%/87.0%	14.0%	99.7%	8.9%
		Annual anti-EBV serology and nasopharyngoscopy	88.9%/87.0%	15.1%	99.8%	9.5%
Yu *et al.* (*Yu et al., 2011*) (Taiwan)	Clin Cancer Res (2011)	VCA IgA (IF) Positive (>1:10 dilution of serum)	36.4%/73.8%	26.2%	99.5%	0.8%
		VCA IgA (ELISA) Positive (OD405 > 0.50)	7.1%/95.7%	4.3%	99.4%	1.0%
		VCA IgA (ELISA) Positive (OD405 > 0.10)	92.9%/16.0%	84.0%	99.7%	0.6%
		EBNA1 IgA Positive (OD405 > 0.20)	50.0%/84.3%	15.9%	99.7%	1.8%
		EBNA1 IgA Positive (OD405 > 0.10)	85.7%/51.2%	49.0%	99.8%	1.0%
		Dnase Positive (>400 neutralizing units)	23.1%/88.1%	12.0%	99.5%	1.1%
		Dnase Positive (>160 neutralizing units)	84.6%/92.3%	8.1%	99.9%	5.6%

Table 4. Comparing different screening seromarkers in high risk NPC screening with positive family history.

5. Conclusion

EBV is associated with the development of NPC and the infection occurs in the early phases of carcinogenesis. A long period of pre-clinical detectable phase offers the opportunity for screening and early diagnosis of NPC. EBV antibodies and EBV DNA have the potential for screening, diagnosis, monitoring and prognosis of NPC. NPC screening in a high-risk, endemic population using EBV-specific serologic markers seems effective. Conduction of prospective randomized controlled screening trials is necessary to validate the cost-effectiveness.

6. Acknowledgment

7. References

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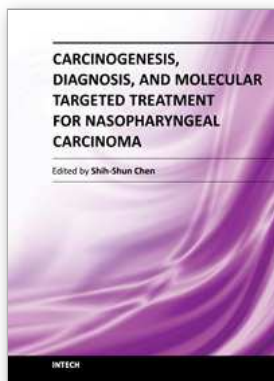
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This book is a comprehensive treatise of the potential risk factors associated with NPC development, the tools employed in the diagnosis and detection of NPC, the concepts behind NPC patients who develop neuro-endocrine abnormalities and ear-related complications after radiotherapy and chemotherapy, the molecular mechanisms leading to NPC carcinogenesis, and the potential therapeutic molecular targets for NPC.

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