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ORIGINAL RESEARCH

Pathology of Nasopharyngeal Carcinoma in Sudanese Patients and its Association with Epstein-Barr Virus: A Report from a Single Center in Khartoum

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Abstract

Objectives: The aim of this study is to describe the pathology of nasopharyngeal carcinoma in Sudanese patients and to investigate its association with the Epstein-Barr virus (EBV).

Study design: This is a prospective descriptive cross-sectional study conducted at the ENT Khartoum Teaching Hospital, Khartoum City, Sudan.

Subjects and methods: Patients with suspected nasopharyngeal carcinoma reporting to our centre between 2006 and 2008 were studied. Biopsy samples from the nasopharynx were obtained from 68 patients suspected to have NPC. Part of the biopsy was fixed in neutral 10% formalin and processed for light microscopy. The other part was not fixed and was used to extract DNA for the detection of EBV genome. The tumours in the formalin-fixed paraffin-embedded biopsies were classified according to the WHO system of classifying NPC. Genomic DNA was extracted from the fresh unfixed biopsies of patients with histologically confirmed NPC and individuals who had other non-NPC lesions or a normal mucosa. The majority of the lesions in the non-NPC cases were adenoids. The EBV genome was detected by PCR using EBNA-1, and LMP-1 primers.

Results: Of the 68 patients studied, 58 had histologically proven nasopharyngeal carcinoma. The tumours were classified as type 2 in 23 patients, type 3 in 32 and mixed types 2 and 3 in 3 patients. EBV genome was detected in 77.6% and 84.5% of the tumours by EBNA-1 and LMP-1 primers, respectively. Of the non-NPC cases, the highest infection with EBV was in patients with adenoids. The virus was detected in 8 of the 44 adenoids (18.2.%) with EBNA-1 primer and in 11 (25%) samples with LMP-1 primer. The significance of these findings is discussed.

Conclusion: In Sudan, EBV is strongly associated with nasopharyngeal cancinoma at a frequency comparable to that in countries with intermediate degree of endemicity for the tumour.

Keywords: Nasopharyngeal carcinoma, Sudan, Epstein-Barr virus, EBNA-1, LMP-1, PCR

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Introduction

Nasopharyngeal carcinoma (NPC) arises from the epithelium that covers the nasopharyngeal mucosa. It was first described by Regaud and Schmincke in 1921.[1] Squamous cell carcinomas account for 97% of malignant neoplasms of the nasopharynx.[2] The tumour has two unique features: the presence of the Epstein-Barr virus (EBV) genome in non-keratinizing NPC tumours[3] and heavy infiltration of the tumor by non-malignant lymphocytes.[4]

According to WHO, NPC is classified pathologically into types 1, 2 and 3.[5] Types 2 and 3, but not type 1, are associated with EBV infection.[6]

Only three reports had previously described the pathology of NPC in Sudan.[7–9]

The study reported here is the first in Sudan to investigate the association between EBV and the pathology of NPC using the polymerase chain reaction (PCR).

Materials and Methods

After informed consent, tissue samples were collected from 68 untreated patients suspected to have primary NPC. The patients were seen at the ENT Department, Khartoum Teaching Hospital. The nasopharynx was examined with a 30° rigid endoscope, and a biopsy sample was taken under direct vision. The sample was divided in two parts; one half was fixed in 10% neutral formalin and processed for light microscopy, while the other half was not fixed and was used to extract DNA for detection of EBV by PCR, using specific primers. The primers used were Epstein-Barr virus Nuclear Antigen-1 (EBNA-1) and Latent Membrane Protein-1 (LMP-1). Five 5 µm sections of the paraffin-embedded tissues were stained with hematoxylin and eosin for histological examination.

Forty four biopsies from patients with adenoids were examined for the presence of EBV using EBNA-1 and LMP-1 primers.

The study was approved by the Ethical Review Committee Board of the Institute of Endemic Diseases, University of Khartoum in March 2005.

Detection of EBV Genome

DNA was extracted with chloroform and precipitated by ethanol. Fresh, unfixed samples from NPC cases and other lesions were suspended in lysis buffer containing 400 mM NaCl, 6M guanidine chloride and 300 µl of 7.5% ammonium acetate and incubated overnight at 37 °C. Chloroform was then added, the supernatant was collected, and DNA was precipitated by ethanol.[10] The quantity of DNA was measured with Nanodrop. We amplified regions of Epstein-Barr virus Nuclear Antigen-1 (EBNA-1) (forward primer: 5′ TGAATACCACCAAGAAGGTG 3 and reverse primer: 5′AGTTCCTTCGTCGGTAGTC 3′) and latent membrane protein-1 (LMP-1) (forward primer: 5′CCGAAGAGGTTGAAAACAAA 3′ and reverse primer 5′GTGGGGGTCGTCATCATCTC 3′) genes for identification of EBV DNA. Amplification of the human growth hormone gene (forward primer 5′GCCTTCCCAACCATTCCCTTA 3′ and reverse primer: 5′TCACGGATTTCTGTTGT GTTTC 3′) provided a marker for the presence of intact genomic DNA.

The amplified DNA was analyzed by electrophoresis on 2% agarose gel, and the product was visualized by ethidium bromide staining.

Statistical Analysis

Statistical analysis of EBV status in relation to histological data was performed with the Chi-square test in SPSS version 15.

Results

Of the 68 suspected cases, 58 proved to be nasopharyngeal carcinoma. The age of the patients ranged from 10 to 80 years, and the median age was 45 years. Males accounted for 74.1% of the patients.

Histopathology

Twenty three (40%), 32 (55%) and 3 (5%) of the biopsies were classified as WHO types 2, 3 and mixed type 2 and 3, respectively; there were no cases of type 1 NPC. Type 2 tumors are characterized by spindle-shaped cells with dark nuclei, small but often inconspicuous nucleoli and scanty tapering cytoplasm; they resembled transitional cell carcinoma of the bladder (Fig. 1). Type 3 tumors were composed of cells with vesicular nuclei, large nucleoli and indistinct cytoplasmic margins. The tumours were rich in small lymphocytes, as shown in Figure 2. Mixed type 2 and 3, is shown in Figure 3.

Figure 4 shows a normal nasopharyngeal mucosa. It consists of lymphoid tissue covered by stratified squamous epithelium. In addition to squamous cells,

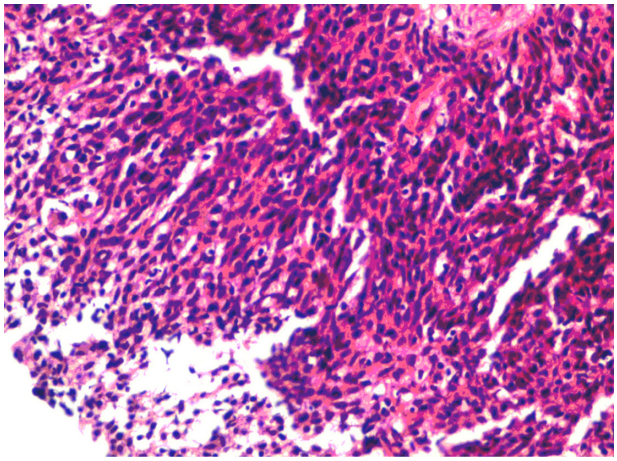

Figure 1. Type 2 NPC. The tumour cells are spindle shaped with elongated dark nuclei and tapering cytoplasm. The tumour resembles transitional cell carcinoma of the urinary bladder (H&E×40).

the normal nasopharynx also contains pseudostratified columnar ciliated epithelium (not shown).

Of the non-NPC and non-adenoid samples, four showed hyperplasia and/or dysplasia of the nasopharyngeal epithelium; two were apparently normal nasopharynx. Four samples were lymphomas. One of the apparently normal biopsies was from a patient who proved to have pleomorphic adenoma of the submandibular salivary gland. The latter was mistaken clinically for enlarged lymph nodes containing metastasis from the nasopharynx. Accordingly a biopsy from the nasopharynx was made. Two of the lymphomas were angiocentric NK/T cell lymphomas.

Detection of EBV

In 58 NPC cases, EBV genome was detected in 45 (77.6%) and 49 (84.5%) cases by EBNA-1 and LMP-1 primers respectively (Figs. 5 and 6). In the 44 adenoids biopsies, the virus genome was found in 8 (18.2%) and 11 (25%) samples by EBNA-1 and LMP-1 primers respectively. Both EBNA-1 and LMP-1 were more significantly associated with NPC than EBV positive adenoids. ($P = 0.000$) (Table 1). None of the histological NPC types was significantly more associated with the virus than the others (Tables 2 and 3).

The normal nasopharyngeal mucosa from the case of salivary gland adenoma, another normal biopsy and two of the lymphoma cases were positive for EBNA-1 and LMP-1 primers. The positive lymphomas were angiocentric NK/T cell lymphomas. The cases showing hyperplasia/dysplasia were negative for EBV.

Because the numbers in this heterogenous group were small, we did not statistically compare the

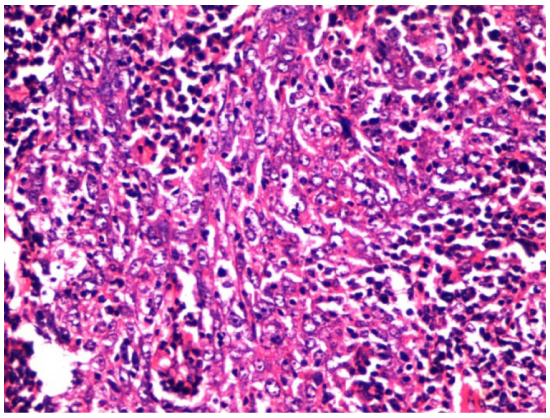

Figure 2. Shows a type 3 NPC. The tumor cells have vesicular nuclei and prominent nucleoli; the cytoplasm has indistinct cytoplasmic borders. The small dark cells are normal lymphocytes (H&E×40).

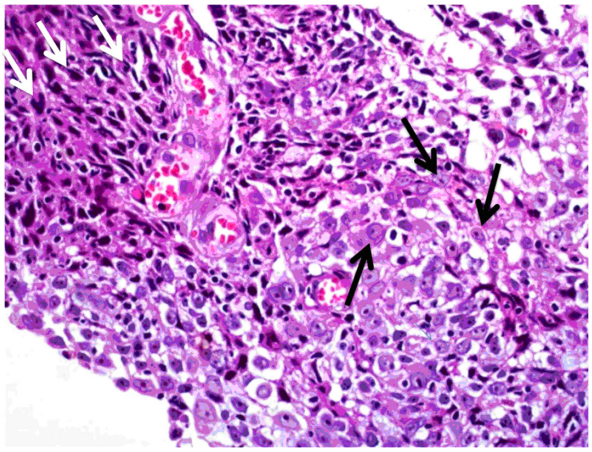

Figure 3. Pathology of mixed type 2 and 3 NPC. The black arrows show type 3 tumour cells. The white arrows show type 2 tumour cells (H&E×40).

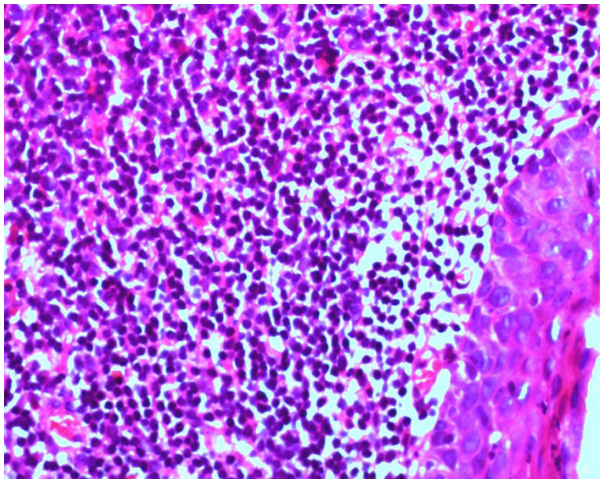

Figure 4. Normal nasopharynx showing a nodule of lymphoid tissue and a lining of stratified squamous epithelium (H&E×40).

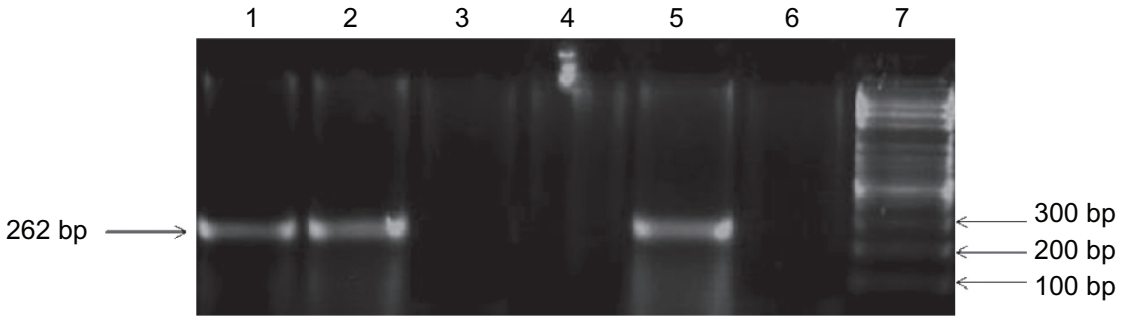

Figure 5. Gel picture showing PCR result of EBNA-1 primer (262 bp). Lane 1 positive control, lane 2, 5 positive samples, lane 3, 4, and negative samples, lane 6 negative control, and lane 7 DNA marker 100 bp.

presence of the virus in the group with that in the NPC cases.

Discussion

In most parts of the world NPC is a rare disease. In the USA and Western Europe, NPC occurs sporadically and is primarily related to the use of alcohol and tobacco.[11]

The sporadic form of NPC is frequently a keratinizing squamous cell carcinoma (WHO type 1).[12] In areas considered endemic for NPC, the tumour is usually WHO type 2 or 3. The endemic areas include the southern parts of China, other parts of Southeast Asia, and the Mediterranean basin.[13] The disease is also prevalent among Alaskan Eskimos.[14]

In endemic areas, the majority of the nasopharyngeal carcinomas are classified as non-keratinizing WHO type 2 or undifferentiated type 3 tumours.[15] In contrast, in non-endemic countries, up to 50% of tumors are of the well differentiated squamous cell variant, (WHO type 1).[16] The majority of our cases were type 2 or 3; only three cases showed a mixed type 2 and 3 pattern. As far as we are aware the mixed type was not reported in the world literature before. It seems that the non-keratinizing tumours originate from a pleuripotential cell, capable of differentiating into clear cut types 2 or 3, but occasionally a mixed pattern of differentiation is encountered. Reasons behind these different patterns of differentiation are not clear.

The pathology of nasopharyngeal carcinoma in Sudan and its association with EBV indicates that it is of the endemic type. This is in agreement with Abuidris et al who noted that the pathology of NPC in Sudan resembles that seen in endemic areas.[9]

Epstein-Barr virus has evolved an effective mechanism for infection, persistence, and spread.[17] It infects more than 90% of the population in early childhood, most often without evident consequence.[18] The virus has been associated with lesions in immunosuppressed patients, in those with polyclonal B cell lymphoproliferative disorders and patients with Burkitt's lymphoma or Hodgkin's disease.[19] However, nasopharyngeal carcinoma shows the strongest worldwide

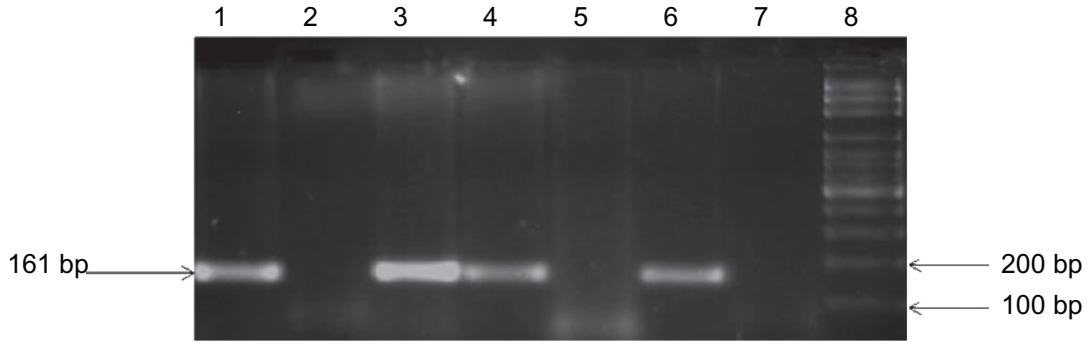

Figure 6. Gel picture showing PCR result of LMP-1 primer (161 bp). Lane 1 positive control, lane 2 and 5 negative samples, lane 3, 4, and 6 positive samples, lane 7 negative control and lane 8 DNA marker 100 bp.

Table 1. Detection of EBV genome by PCR in NPC and adenoids samples using EBNA-1 and LMP-1 primers.

Primer	Adenoids	NPC cases	*P* value
EBNA-1			
Positive	8	45	0.000
Negative	36	13	
LMP-1			
Positive	11	49	0.000
Negative	33	9	

Note: Chi-square test is significant when *P* value is < 0.05.
Abbreviations: NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus; EBNA-1, EBV nuclear antigen-1; LMP-1, latent membrane protein-1.

association with the virus.[20] EBV genome is detected in all histological types of NPC from high-incidence areas.[21] This even includes WHO type 1 cases in these areas.[22]

In the present study EBV genome was detected in 77.5% and 84.1% of all NPC biopsies with EBNA-1 and LMP-1 respectively. These figures are similar to those reported from New Zealand.[21] Sbih-Lammali et al showed that 90% of NPC cases from North Africa are EBV associated types 3 and 2.[23] Krishna et al found EBV genome in 69% of cases from India.[24] Sheen et al detected EBV genome in 91.4% of cases in Taiwan.[25] All these reports are from high or intermediate endemic areas. The presence of the virus in the different histological types in our cases is in agreement with findings in other ares of comparable endimecity for the tumour.[26,27]

Ideally, we should have included biopsies from a negative control group consisting of normal individuals of the same age and sex as the NPC patients. This was not possible. Instead we used tissues from patients with other nasopharyngeal lesions and two patients with apparently normal mucosa. This was undertaken

Table 2. Detection of EBV using EBNA-1 primer in different histological types.

Histopathology	EBNA-1 positive	EBNA-1 negative
Type 2	18	5
Type 3	14	8
Mixed type 2 and 3	3	0
Total	45	13

Note: *Chi-square test. *P* value is 0.60.

Table 3. Detection of EBV using LMP-1 primer in different histological types.

Histopathology	LMP-1 positive	LMP-1 negative
Type 2	20	3
Type 3	26	6
Mixed type 2 and 3	3	0
Total	49	9

Note: *Chi-square test. *P* value is 0.63.

in order to evaluate the significance of the presence of EBV in nasopharyngeal lesions other than NPC. The highest frequency of EBV infection in non-NPC cases was in patients with adenoids. Others reported EBV in 72% of adenoids removed from children aged 3–15 years.[28]

The two EBV positive lymphomas in this report were examples of angiocentric NK/T cell lymphoma which, is known to be associated with this virus.[29] The gold standard for the diagnosis of NPC is the histopathological demonstration of the tumour in biopsies of the nasopharynx or in metastases of the tumour at distant sites. The detection of EBV by serology or in nasopharyngeal biopsy, in the absence of histologically documented tumour, is not diagnostic of nasopharyngeal carcinoma. Patients with positive EBV infection but whose nasopharyngeal biopsies are negative for NPC should be re-evaluated clinically and followed up closely for the possible development of the tumour in the future.

In conclusion, the histopathology of NPC in Sudan is of the types described in areas endemic for the disease and is significantly associated with EBV infection.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors

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References

1. Schmincke A. On lymphoepithelial tumors. *Beitr Pathol Anat.* 1921;68: 161–70.
2. Yeh S. The relative frequency of cancer of the nasopharynx and accessory sinuses in Chinese in Taiwan. In: Muir CS, Shanmugaratnam K, editors, Cancer of the Nasopharynx. UICC Monograph Series 1, Copenhagen: Munksgaard; 1967:54–7.
3. Desgranges C, Wolf H, de-The G, et al. Nasopharyngeal carcinoma. X. Presence of Epstein-Barr genomes in separated epithelial cells of tumours in patients from Singapore, Tunisia and Kenya. *Int J Cancer*. 1975;16:7–15.
4. Herait P, Ganem G, Lipinski M, et al. Lymphocyte subsets in tumor of patients with undifferentiated nasopharyngeal carcinoma: presence of lymphocytes with the phenotype of activated T cells. *Br J Cancer*. 1987;55:135–9.
5. Wei WI, Sham JS. Nasopharyngeal carcinoma. *Lancet*. 2005;365:2041–54.
6. Neel HB 3rd, Pearson GR, Taylor WF. Antibodies to Epstein-Barr virus in patients with nasopharyngeal carcinoma and in comparison groups. *Ann Otol Rhinol Laryngol*. 1984;93:477–82.
7. El Hassan AM, Nilosev B, Daoud EH, et al. Malignant discases of the upper respiratory tract in the Sudan. In: Clifford P, Linsell CA, Timms GL, editors. Cancer in Africa. Nairobi: East African Publishing House; 1967:307–13.
8. Hidayatalla A, Malik MO, El Hadi AE, et al. Studies on nasopharyngeal carcinoma in the Sudan—I. Epidemiology and aetiology. *Eur J Cancer Clin Oncol*. 1983;19:705–10.
9. Abuidris DO, Elgaili EM, Elhaj AH, et al. Histopathological patterns of nasopharyngeal carcinoma in Sudan. *Saudi Med J*. 2008;29:962–5.
10. Wu Q, Chen M, Buchwald M, et al. A simple, rapid method for isolation of high quality genomic DNA from animal tissues. *Nucleic Acids Res*. 1995;23:5087–8.
11. Vokes EE WR, Lippman SM, Hong WK. Medical progress in head and neck cancer. *N Engl J Med*. 1993;383:184–94.
12. Vokes EE, Liebowitz DN, Weichselbaum RR. Nasopharyngeal carcinoma. *Lancet*. 1997;350:1087–91.
13. Chan ATC, Teo PML, Johnson. Nasopharyngeal Carcinoma. *Annals of Oncology*. 2002;13:1007–15.
14. Henderson S, Rowe M, Gregory C, et al. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell*. 1991;65:1107–15.
15. Altun M, Fandi A, Dupuis O, et al. Undifferentiated nasopharyngeal cancer (UCNT): current diagnostic and therapeutic aspects. *Int J Radiat Oncol Biol Phys*. 1995;32:859–77.
16. Peters LJ, Batsakis BJ, Goepfert H, et al. The diagnosis and management of nasopharyngeal carcinoma in Caucasians. *In* Textbook of uncommon cancer. KJ. Williams CJ, Green MR, Raghavan D, editors. Wiley and Sons Ltd, New York; 1988:975–1006.
17. Miller G. Epstein-Barr virus: biology, pathogenesis, and medical aspect. In: Fields BN, Knipe DM, editors. Virology. 2nd ed. New York: Raven Press; 1990:1921–58.
18. Benchimol S MM. Virus oncogens and tumor suppressor genes. In: Tannock IF HR, editor. The Basic Science of Oncology. New York: McGraw-Hill; 1998:79–107.
19. Niedobitek G, YL. Epstein-Barr virus persistence and virus-associated tumours. *Lancet*. 1994;343:333–5.
20. Pathmanathan R, Prasad U, Sadler R, et al. Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. *N Engl J Med*. 1995;333:693–8.
21. Cohen J. Epstein-Barr virus infection. *N Engl J Med*. 2002;343:481–92.
22. Popat SR, Liavaag PG, Morton R, et al. Epstein Barr virus genome in nasopharyngeal carcinomas from New Zealand. *Head Neck*. 2000;22:505–8.
23. Sbih-Lammali F, Djennaoui D, Belaoui H, et al. Transcriptional expression of Epstein-Barr virus genes and proto-oncogenes in north African nasopharyngeal carcinoma. *J Med Virol*. 1996;49:7–14.
24. Krishna SM, James S, Kattoor J, et al. Serum EBV DNA as a biomarker in primary nasopharyngeal carcinoma of Indian origin. *Jpn J Clin Oncol*. 2004;34:307–11.
25. Sheen T, Ko J, Chang Y, et al. Nasopharyngeal carcinoma swapand PCR for the screening of nasopharyngeal carcinoma in endemic area: A good supplement to the serologic screening. *Head Neck*. 1998;20:732–8.
26. Dickens P, Srivastava G, Loke SL, et al. Epstein-Barr virus DNA in nasopharyngeal carcinomas from Chinese patients in Hong Kong. *J Clin Pathol*. 1992;45:396–7.
27. Chang Y, Tyan Y, Liu ST, et al. Detection of Epstein-Barr Virus DNA sequences in nasopharyngeal carcinoma cells by enzymatic DNA amplification.
28. Endo LH, Sakano E, Camargo LA, et al. The EBV action in tonsils and adenoids. *International Congress Series*. 2003; 1257:263–7.
29. Davidson SP, Habermann TM, Stricker JG, et al. Nasal and Nasopharyngeal Angiocentric T-Cell Lymphoma. *Laryngoscope*.1996;106:139–43.