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The Role of EBV Markers in Diagnosis, Treatment and Monitoring of Nasopharyngeal Carcinoma in Jakarta, Indonesia

ACADEMISCH PROEFSCHRIFT

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Marlinda Adham

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		dr. A.E. Greijer

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Chapter 1

Introduction and Brief Outline of the thesis

GENERAL CHARACTERISTICS OF NASOPHARYNGEAL CARCINOMA

Anatomy of the nasopharynx and the lymphogenic spread of nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a rare malignancy in most parts of the world and it is one of the most confusing, commonly misdiagnosed and poorly understood diseases.

The nasopharynx (NP) is defined as the part of the pharynx, which lies behind the nasal fossa and extends inferiorly as far as the level of the soft palate (Fig.1). The NP is a hollow, air-containing passageway. The posterior wall occupies the angle between the base of skull above and the vertebral column. Anterior, the NP communicates with the nasal cavity through the posterior nares or choanae. The lower part of the anterior wall is formed by the soft palate. The lateral wall is formed by the superior constrictor muscles. The Eustachian tube ostium is situated in the lateral wall of the NP and the lateral pharyngeal recess or fossa of Rossenmuller[1,2]. In this area stratified squamous and ciliated epithelia meet. This is called the transitional zone and is liable to metaplastic and neoplastic changes.

It has been shown that opening of the Eustachian tube is dependent on the action of the musculus tensor veli palatini. Interference with its action by tumor infiltration is associated with tubal dysfunction and middle ear problems with hearing loss, which is an early sign of NPC. Its inaccessible location underlines the problem of the otolaryngologist in performing a thorough examination of the area.

The NP is the side of marked aggregation of lymphoid tissue and forms part of a lymphoid ring i.e the Waldeyer's ring. The lymphatic drainage system is correspondingly extensive. A dense capillary network in the mucosa exists throughout the pharynx and gives origin to three main groups of sub-mucosal collecting trunks. The retropharyngeal space needs some more attention in that it contains the median and lateral groups of retropharyngeal lymph nodes including the node of Rouviere. A good understanding of this extensive lymphatic system is of great importance in tumor staging and management of tumor spread and it should be noted that lymphatic channels do cross the midline[3,4]

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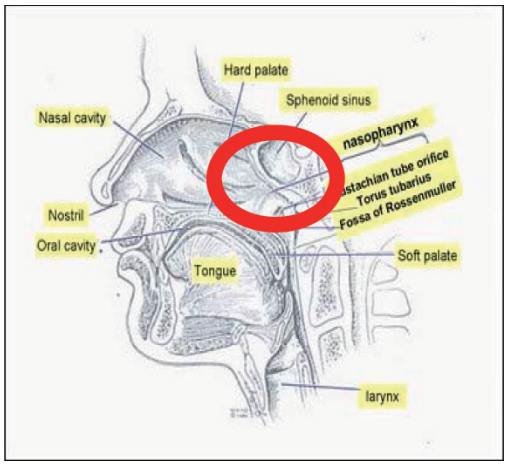

Figure 1 | The Anatomy of nasopharynx and connection with other structures in the back of the nasal cavity[4]

Epidemiology of the tumor

NPC has a remarkable racial and geographical distribution with complex interactions of genetic, viral, environmental and dietary factors.

There was an estimated incidence of 84,400 cases of NPC and 51,600 deaths in 2008, representing about 0.7% of the global cancer burden. This disease may be considered as one of the rarer cancer forms globally, ranking as the 24th most frequently diagnosed cancers worldwide and 22nd within the developing world[5].

NPC is a relatively rare malignancy in most parts of the world. It is more frequent in males than females in both the developing and developed world, with incidence rates commonly 2 to 3 times higher in males in higher resource countries. It accounts for 2% of all head and neck squamous cell carcinomas, with and incidence of 0.5 to 2 per 100,000 in the United States[6]. However, it is endemic in many geographical regions, including Southern China, Southeast Asia, Indonesia, Japan, the Middle East/North Africa and Eskimos in arctic regions[3,6–8]. Some North-East parts of India have a high incidence of NPC as well. Ho et al. reported that NPC is the third most common malignancy among men, with an incidence of 50 per 100,000 in the Guangdong province of Southern China[9].Some references report much higher incidences of 50-150 cases per 100,000 in Southern China, particularly in Hong Kong and Guangzhou (formerly known as Canton, and the capital of the province of Guangdong). Indeed, this malignancy is often referred to as “Cantonese cancer” or “Kwangtung tumor”[10–12]. Emigration from high to low incidence areas such as the United States and Canada reduces

the incidence of NPC in first generation Chinese, but it still remains at seven times the rate compared to Caucasians[13,14]. Chinese of southern origin have a uniquely high risk up to 10-20 /100,000 for males resulting in around 20.000 new cases yearly in China. The highest recorded NPC incidence is found in the Bidayu people of Borneo Island (Kalimantan), with an age-adjusted incidence of 35/100,000[15]. In Indonesia it is the most frequent cancer in the head and neck and ranks as number 4 in males with an incidence of 6.2/100,000 and has been added to the Globocan overview of WHO-IARC only recently, because of prior lack of specific diagnostic facilities and data[16]. The epidemiology and etiology specifically for Indonesia will be discussed in detail in chapter 2[17].

Etiology and risk factors

NPC presents as a complex disease caused by an interaction between chronic infection with an oncogenic gamma herpes virus, the Epstein-Barr virus (EBV) and environmental and genetic factors, involving a multistep carcinogenic process[6]. EBV exists worldwide, infecting over 95% of the global adult population[18]. In Hong Kong, 80% of the children are infected by 6 years of age, and almost 100% have seroconverted by 10 years of age[19]. Although primary EBV infection is typically subclinical, the virus is associated with the later development of several malignancies including NPC[7]. It is transmitted by saliva, and its primary infection occurs during childhood with replication of the virus in the oro-pharyngeal lining cells, followed by a latent infection of B lymphocytes (primary target of EBV). Epstein-Barr virus initiates an early active (or lytic) infection; the virus then persists in a latent state until it is reactivated under certain conditions of immunosuppression or illness. Elevated titers of EBV-associated antigens (especially of IgA class), a latent EBV infection identified in neoplastic cells of virtually all cases of NPC, the clonal EBV genome consistently detected in invasive carcinoma and high-grade dysplastic lesions suggest a critical role of EBV in the pathogenesis of NPC in endemic areas[6].

Environmental factors and dietary habits are also reported to be related to NPC. Salted fish consumption in early childhood has been correlated with an unusually high incidence of nasopharyngeal cancer in the boat communities of Hong Kong's harbors[12,20]. N-nitrosodimethylamine in salted fish, perhaps in combination with vitamin deficiency, has been considered a likely carcinogen[10,12,21]. The process of salt preservation is inefficient, allowing fish and other foods to become partially putrefied. As a result, these foods accumulate significant levels of nitrosamines, which are known to be carcinogenic in animals[22]. Salt preserved fish also contain bacterial mutagens, direct genotoxins, and EBV-reactivating substances[23,24], any or all of which could also contribute to the observed association.

Occupational hazards, including exposures to formaldehyde, dust and smoke particles and certain aromatic hydrocarbons, have been investigated as risk factors for nasopharyngeal cancer[25–27]. Formaldehyde is a recognized nasal cavity carcinogen in rodents. Smoke particles from incomplete combustion of coal, wood, and other materials are also of the size and weight to be deposited mostly in the nasopharynx[28]. Several studies conducted in high-and low-risk populations during the past decade have obviously indicated the nasopharynx as a tobacco susceptible cancer site[20,29], and that exposure to parental smoking during childhood plays a role. Ever smokers exhibit a roughly 30%-100% excess risk compared with life-long non-smokers[30]. In low risk populations, data on risk factors are scarce. Also, a proportion of cases of NPC are of the differentiated type (WHO type I) and the etiology of these tumors is different from that of the undifferentiated nasopharyngeal cancer. A case-control study conducted in the USA on 231 cases and 246 controls revealed that only differentiated NPCs (118 cases) were clearly related to heavy drinking and tobacco. A strong dose response relationship between cigarette smoking and the risk of differentiated squamous cell carcinoma was observed[31,32]. The use of certain Chinese medical herbs has been suggested to increase the risk for NPC by reactivating EBV infection in the host[33].

A genetic predisposition is suggested by a high incidence of NPC in patients with specific histocompatibility complex profiles, including HLA-A2, HLA-B46 and HLA B58. AW19, BW46 and B17 have also been reported to be associated with an increased risk, whereas HLA-A11 is associated with a decreased risk[34–36]. In rare familial cases, inherited genetic alterations could be the first “hit” and EBV infection may contribute to the second “hit”. Therefore these familial cases usually occur with a younger age of onset[37]. The finding of translocation, amplification and deletion of 3p, 5p and 3q indicates that genetic abberations are possibly contributing to NPC development[38,39].

Viral infection by EBV together with environmental co-carcinogens rather than genetic predominance is believed to be the strongest etiological forces for the development of NPC.

Histopathology

According to WHO classification, NPC is histopathologically divided into 3 categories i.e. keratinizing squamous cell carcinoma (type 1), non-keratinizing carcinoma (type 2), and undifferentiated carcinoma (type 3). The pathology of the different NPC types is presented in figure 2. EBV is more deeply related to the undifferentiated type of carcinoma. This type has a better prognosis than the differentiated non-keratinizing and keratinizing types of carcinoma[1,20]. This is related to the higher (chemo-) radiosensitivity of undifferentiated

carcinomas. Undifferentiated NPC has a higher local tumor control rate, despite a higher incidence of distant metastasis compared to differentiated carcinomas[1,40].

A "Working Formulation Classification" based on the degree of anaplasia and pleomorphism of different cell types was suggested by Yeh[41]. Tumors with cells with marked nuclear hyperchromatism and or evident variation in nuclear size were designated as Type A, whereas those with little to moderate pleomorphism and hyperchromatism were designated as Type B[42].

Both cell types and the degree of anaplasia reflect important prognostic significance and further impact on the patient's outcome. Tumors with evident anaplasia and or pleomorphism (Types A) have a significantly less favorable outcome with a 5 year survival of 30-40% than their counterparts with mild anaplasia (Types B) with a 5 year survival of 60-72%[42].

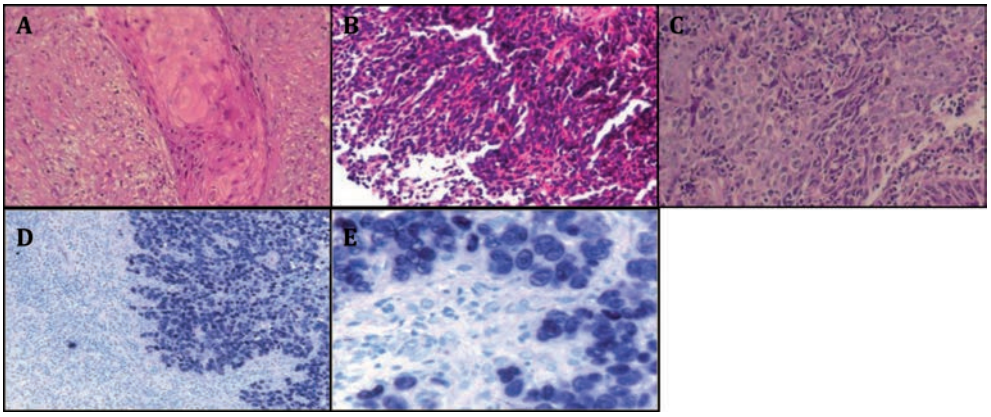

Figure 2 | Histopathology pictures of different Types of WHO classification (Courtesy of Lisnawati Rachmadi. MD Path, her own work in Histopathology UI Jakarta) **A:** Nasopharyngeal keratinizing squamous cell carcinoma, with clear squamous differentiation (WHO-1). The island of tumor shows invasion into the stroma. The tumors cells show obvious squamous differentiation and keratinization and round prominent nucleoli and eosinophilic cytoplasms. **B:** Nasopharyngeal non-keratinizing differentiated carcinoma, with little squamous differentiation (WHO-2). The tumor cells show differentiation with some cytokeratin expression, but in which squamous differentiation is not evident by light microscopy. **C:** Nasopharyngeal non-keratinizing, undifferentiated carcinoma, without squamous differentiation (WHO-3). The tumor cells in syncytial arrangements show round nuclei, pleomorphic, vesicular with prominent nucleoli and spindle cell with dark staining nuclei and inconspicious nucleoli. **D and E:** EBER-RISH showing positive expression in nuclei of NPC tumor cells in the routine paraffin-embedded histopathology specimen.

Published data indicate a higher proportion of keratinizing squamous cell carcinoma among all NPC cases in non-endemic compared with endemic areas. Some studies reported that WHO type 1 accounts for approximately 25% of all NPC in North America, but only 1% in endemic areas; whereas undifferentiated carcinoma, WHO type 3, accounts for 95% of all cases in high incidence areas and 60% of cases in North America[1,22,43].

Clinical Presentations

Wei and Sham[1] divided symptoms presented by NPC patients into four categories (1) symptoms caused by the presence of a tumor mass in the nasopharynx (epistaxis, nasal obstruction, and discharge), (2) symptoms associated with dysfunction of the Eustachian tube (hearing loss), (3) symptoms associated with the superior extension of the tumor (headache, diplopia, facial pain, and numbness), and (4) neck masses. The clinical appearance of NPC is depicted in figure 3. Since the nasopharynx has an abundant supply of regional lymphatic vessels, metastases are frequently found. Cervical lymphadenopathy is often the only clinical manifestation of NPC in patients. Because symptoms, related to NPC in the early stage, are usually nonspecific, most NPC patients are diagnosed in advanced stage. Spread to the neck nodes occurs in a predictable manner; upper levels first, mid jugular and supraclavicular chains later[1,4,44]. Paulino et al. found that a unilateral neck mass was the most common presenting sign, occurring in 80%[45] of the patients.

Cranial nerve involvement, subsequent to invasion of the skull base is seen in 25% of cases[46]. The two principle cranial nerve syndromes associated with nasopharyngeal carcinoma are the ***retroparotid syndrome*** (involving cranial nerves IX, X, XI and XII) and the ***petrosphenoid syndrome*** (involving cranial nerves III, IV, V, and VI). Occasionally, cranial nerve II becomes involved through the foramen lacerum[43]. Isolated cranial nerves most commonly affected was the III, V, VI and XIIth nerves, with symptoms of diplopia, trigeminal neuralgia and or deviation of the tongue[1,44]. Headache can be explained by extension into sphenoid, middle cranial fossa and intracranial extension.

NPC produces its clinical features by invading adjacent structures locally and spreading to neck nodes regionally. Extension of local spread is associated with adverse prognosis. The presence of bulky cervical lymphadenopathy is predictive for distant metastasis. Lung, followed by bone, are the most common sites for metastasis[11].

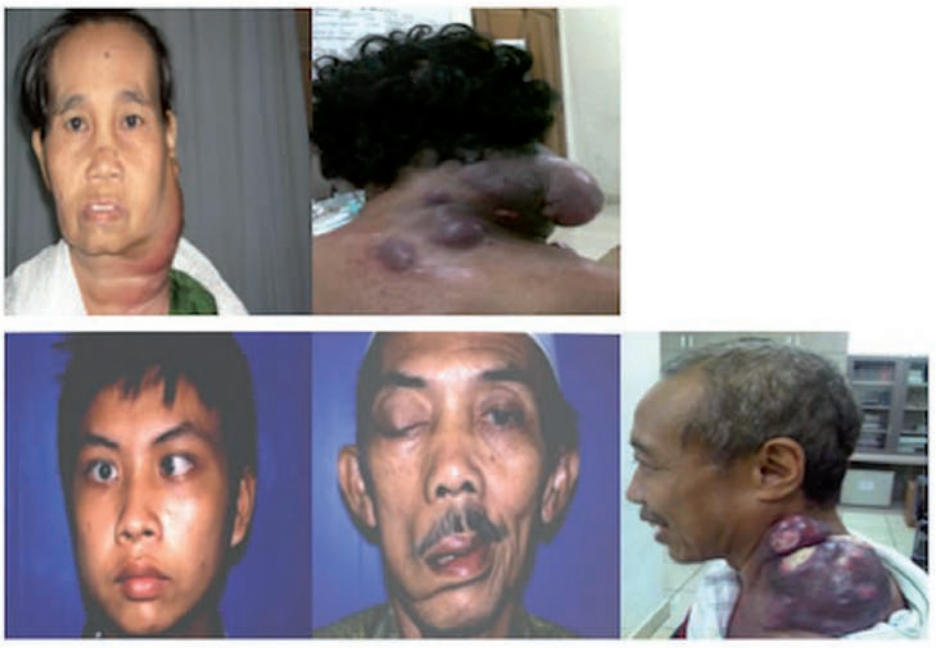

Figure 3 | Clinical appearance of NPC: Enlargement of lymph nodes, and involvement of cranial nerve 3,4,6 that caused diplopia , ptosis and lagophtalmus. All patients gave permission for publication.

Diagnosis and staging

Diagnosis of nasopharyngeal carcinoma is primarily based on the history, physical examination and imaging. For definitive diagnosis a biopsy of the lesion is required. Biopsy can be performed in the office or in the operating room by rigid or flexible endoscope and has a high specificity (99.6%)[2]. Endoscopy gives adequate information of the local status of the disease. Endoscopic evaluation gives the possibility to take a direct biopsy of the lesion for histopathological examination. As long as the tumor involves the mucosa it will be visible by direct endoscopy. Sub-mucosal tumor extension is more difficult to evaluate by endoscopy (Fig.4) due to the fact that the extension of the lesion is hardly visible. Imaging is indispensable for this reason[1,47].

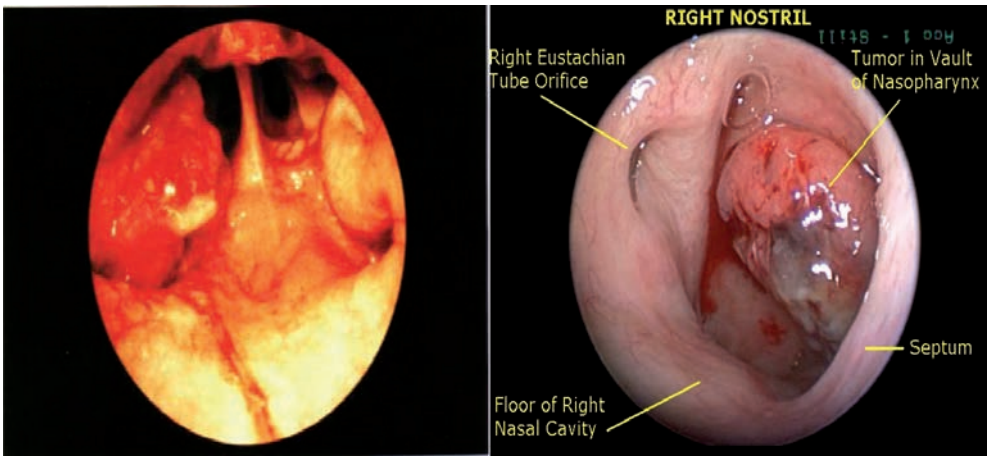

Figure 4 | Endoscopic appearance shows tumor involvement of the fossa of Rosenmuller, obstruction of the Eustachian tube and extension into the nasal cavity through the choanae (left picture), and tumor from the left side of nasopharynx involved the roof of nasopharynx with extension to the right side of nasopharynx[4].

The primary tumor extent should be evaluated by CT scan and/or MRI. MRI is the preferred modality of choice for the imaging of NPC[48,49]. MRI is more sensitive than CT scan for the detection of the extension of the primary tumor. It can reveal soft tissue extent, regional nodal metastasis and perineural growth and may help to depict subclinical NPC missed at endoscopy. MRI is better than CT in displaying both superficial and deep nasopharyngeal soft tissue and for differentiating tumor from normal tissue. MRI is also more sensitive for assessment of retropharyngeal and deep cervical nodal metastases and it can detect bone marrow infiltration[11,50–52]. Bone marrow infiltration is associated with an increased risk of distant metastases[53]. CT scan is considered as a better tool for defining bone erosion[1]. CT has been used for long in staging NPC (Fig.5), especially for the detection of skull base tumor involvement with lytic or sclerotic lesions[54,55], but now it has been largely replaced by MRI for primary tumor and nodal staging. However, CT scan is still used for radiotherapy planning and, in some centers, it is used together with PET using 18F-FDG. PET/CT scan has been shown to be of great value in NPC staging, where the main advantage is for the detection of distant metastasis[56]. It is also used for monitoring patients post-treatment and detecting NPC recurrence. In case a PET scan is not available, chest radiography, hepatic ultrasonography and bone scanning can be used in the assessment of metastatic disease and, ultimately, the staging of this malignancy[57].

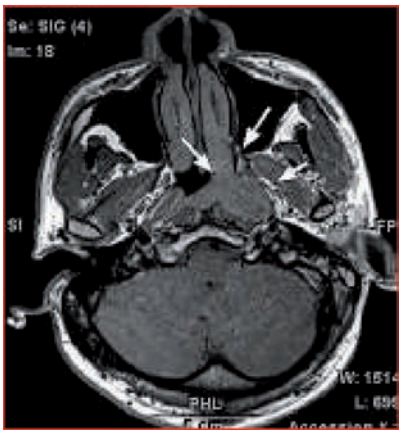

Figure 5 | T1 (former T2a) Nasopharyngeal Carcinoma: tumor from the Left Fossa of Rosenmuller spreading to the nasal cavity

Clinical staging of the tumor represents 4 stages (Table 1) According to the AJCC 2010 7th edition, stage I is T1N0, local disease in soft tissue only. Stage II represents stage I with N1 disease, which is uni or bilateral- retropharyngeal nodes, or unilateral upper neck nodes not greater than 6 cm, or primary tumor extension (T2) into the parapharyngeal region. Stage III is stage II plus N2 disease, which is bilateral upper neck nodes ≤6 cm, or local tumor invasion (T3) with minimal bone invasion or into the paranasal sinus. Stage IV is stage III with N3 disease, which is nodal involvement in the supraclavicular fossa, or lymph nodes greater than 6 cm, or local disease (T4) with cranial nerve involvement, extension to intracranial, or to the hypopharynx, infratemporal fossa, orbit or oropharynx [58,59]. In comparison to the 6th edition a few changes have been made[60]. First, patients with nasal cavity or oropharynx involvement who were classified as T2a in the 6th edition are now classified as T1 and T2b, which in case of parapharyngeal extension has become T2. Second, the role of retropharyngeal lymph node is clarified in the staging system, and (uni- or bilateral) retropharyngeal lymph node invasion is staged as N1. Third, the addition of the term ‘‘masticator space’’ (as a synonym for infratemporal fossa), which was introduced in the 6th edition, has been abandoned, and anatomic masticator space involvement is staged as T4.

Table 1 | Classification criteria and stage grouping by different systems. Changes in the AJCC/UICC staging system (7th edition) for nasopharyngeal cancer[61]

AJCC/UICC 5–6th edition	AJCC/UICC 7th edition
T-category	
T1: Nasopharynx	T1: Nasopharynx, oropharynx, or nasal fossa
T2: Oropharynx or nasal fossa	T2: Parapharyngeal extension
T2a: without parapharynx	
T2b: with parapharynx	
T3: Bony structure, paranasal sinuses	T3: Bony structure, paranasal sinuses
T4: Intracranial extension, cranial nerve, hypopharynx, orbit, infratemporal fossa (masticatory space)	T4: Intracranial extension, cranial nerve, hypopharynx, orbit, infratemporal fossa (masticatory space)
N-category	
N0: None	N0: None
N1: Unilateral node, 6 cm, above supraclavicular fossa	N1: Unilateral cervical, uni/bi-lateral retropharyngeal, 6 cm, above supraclavicular fossa
N2: Bilateral node, 6 cm, above supraclavicular fossa	N2: bilateral cervical node, 6 cm, above supraclavicular fossa
N3a: >6 cm	N3a: >6 cm
N3b: in supraclavicular fossa	N3b: in supraclavicular fossa

Narrow-band imaging (NBI) endoscopy is a promising tool to differentiate non-malignant from malignant nasopharyngeal lesions on the basis of the morphologic findings of mucosal capillary vessels in vivo. In addition, NBI may increase the diagnostic value of endoscopy in populations at high risk for NPC[62].

For diagnosis and typing, EBER in situ hybridization is the most reliable method for determining if a lesion is EBV associated and considered as the gold standard for detecting and localizing latent EBV in tissue samples[63].

Treatment of NPC

NPC is more sensitive to radiotherapy and or chemotherapy than other head and neck cancers. The 5 years overall survival rates for stage I, II, III and IV disease is 90%, 80%, 70% and 50% respectively. Since NPC is highly radiosensitive, radiotherapy (RT) has always been the main treatment of choice for this cancer. The major limitations of 2D planning for nasopharyngeal carcinoma can now be overcome with 3D conformal radiotherapy and intensity-modulated radiotherapy (IMRT)[64,65]. The main aim of using 3D-CRT (Conformal Radiation therapy) and IMRT is to produce isodose curves with high conformity for the target volume, while decreasing the dose into surrounding tissues. Consequently, acute and late morbidity will decrease, while increasing the dose to the target volume. Prophylactic

neck treatment is recommended for NPC without clinical neck nodes, because 30% - 40% of these patients will develop regional disease if the neck is not treated.

For early stage disease (T1N0M0), radiotherapy is given to the nasopharynx and the neck[1,6]. For advanced stage (T1N1-3M0 and T2-4N0-3M0), chemoradiation is the treatment of choice. Chemotherapy can be administered as neoadjuvant or concurrent to radiation. The standard agent used in concurrent chemoradiotherapy is cisplatin. This provides a benefit in terms of overall survival and on both loco-regional and distant control[66–72]. While three cycles of adjuvant cisplatin-5FU has been a standard part of many concurrent chemoradiotherapy regimens, the actual benefit of the adjuvant cycles are uncertain and toxic effect is substantial[73]. Cisplatinum based induction chemotherapy has been shown by some studies to improve disease-free survival and may be considered in locally advanced disease although it is not seen as a standard treatment.[74]

Radiotherapy is applied with a dose of 65-75 Gy to the primary tumor and 65-70 Gy to the involved neck nodes, whereas the dose for prophylactic treatment for the node-negative neck is 50-60 Gy. This treatment has successfully controlled T1 and T2 tumors in 75-90% of cases and T3 and T4 tumors in 50-75% of cases[61,75–78]. For T1 and T2 tumors, a booster dose by use of intracavitary brachytherapy can improve tumor control by 16%[79].

Nodal control is achieved in 90% of N0 and N1 cases, but the control rate drops to 70% for N2 and N3 cases[74]. Evaluation is performed with CT scan or MRI, ultrasound of the neck and endoscopy of the nasopharynx 8-12 weeks post treatment to assess the tumor response. For patients with distant metastasis, platinum based combination of chemotherapy or radiotherapy for brain metastasis and weight bearing bones can be considered.

To minimize the risk of late toxicity (particularly, to adjacent neurological structures), fractional dose >2 Gy per day and excessive acceleration with multiple fractions >1.9 Gy/ fraction should be avoided. IMRT may offer improvement in local tumor control, and reduction in radiation xerostomia in early-stage disease.

It is well demonstrated that once radiotherapy has started the full course of radiation should be given in 45-47 days, due to the fact that the number of clonogenic cells in cycle will increase once radiotherapy is started. Interruptions have been shown to be hazardous for the expected outcome of radiotherapy, due to a phenomenon known as accelerated repopulation which occurs in the tumor tissue[80].

Complications of treatment are hearing deficits due to chemotherapy, reduced smell and vision due to decreased function of olfactory and optical nerve, vascular stenosis and or

induced malignancy by the radiotherapy it self. Radiotherapy also effects dental hygiene, due to a reduction of the function of the salivary glands.

Follow-up after primary treatment

The modalities commonly used in the follow up of patients with NPC include clinical examination, endoscopy and imaging. Inspection with a flexible or rigid endoscope plays a primary role in follow up examination. However, mucosal reactions after radiotherapy make it difficult to find early recurrent lesions. Secretions and crust covering the nasopharyngeal mucosa also hamper the early detection of local recurrence. Detection of sub-mucosal or deep-seated recurrent lesions is difficult with endoscopic examinations only.

For the imaging after initial treatment, CT and MRI are widely used for the detection of recurrent or residual lesions. MRI is superior to CT in the detection of soft tissue abnormalities. The baseline MRI or CT study is conducted 2 to 3 months after termination of the initial treatment. After the baseline evaluation, close follow up is recommended every 3 to 6 months for the first 2 years post-treatment[81]. FDG-PET in the detection of residual or recurrent NPC lesions has been reported from several institutes. FDG-PET is increasingly being used for detection of recurrent lesions and may distinct tumor from post irradiation changes, such as tissue necrosis, fibrosis, and edema[82–84]. Liu reported that sensitivities for CT, MRI, PET for the detection of residual or recurrent NPC lesions were 76, 78, and 95% respectively[85]. This suggests that PET can be a useful tool for the detection of recurrent or residual NPC lesions, but there are also limitations with the use of PET for the detection of early recurrent NPC lesions. FDG uptake can be increased by inflammatory reactions in the early period after radiotherapy[85]. For the detection of distant metastases, the use of serum EBV DNA has been shown to be more sensitive and reliable than other options[86].

Narrow Band Imaging (NBI) is a novel technique that enhances the diagnostic sensitivity of endoscopes for characterizing tissues using narrow-band width filters in a sequential red-green-blue illumination system. Superficial mucosal carcinoma lesions, which are rarely detected using conventional endoscopy, can be observed with NBI by viewing the non-angiogenic, microvascular proliferation pattern[87,88] (Adham ongoing study). Lin and Wang[88] reported that early recurrent lesions of NPC after radiotherapy were successfully detected by NBI coupled with conventional endoscopy.

Treatment of locoregional recurrent and persistent NPC

Based on retrospective analysis on diagnosis of recurrent regional disease, the recommended procedure after curative intent chemo-radiotherapy is a FNAC and CT scan. When positive

nodes are detected, radical neck dissection is the preferred treatment. When the FNAC is negative or inconclusive, excision biopsy of the affected node should be done, preferably with frozen sections in combination with neck dissection if malignant cells are found.[89] Radical neck dissection offers better results than re-irradiation. The overall 5 years survival rate for treatment with radiation is around 20%[46]. Radical neck dissection as salvage procedure has achieved a 5 years tumor control rate of 66% in the neck and a 5 year actuarial survival of 38%[90]. When tumor in the neck node extends beyond the confines of the lymph node, brachytherapy could be applied to the tumor bed in addition to radical neck dissection[1].

Locally persistent and recurrent tumors after radiotherapy or chemo-radiation can be addressed by re-irradiation, brachytherapy, stereotactic radiotherapy, photodynamic therapy or surgically. Surgery can be performed either through a minimal invasive (endoscopic) approach, or with open surgery by a mandibular or maxillary swing procedure or infratemporal approach from the lateral aspect,[91,92], transpalatal, transmaxillary, and transcervical approaches from the inferior aspect[93,94]. Surgery remains a challenge due to the hidden position of the nasopharynx[95]. The 5 year actuarial control of tumors in the nasopharynx is about 65% and the 5 year disease free survival rate is around 54%[96,97]. Nasopharyngectomy is usually offered when there is only evidence of local recurrence or persistent disease. [91,92,98,99].

Re-irradiation can also be used as therapy for local recurrent NPC, although its use is limited by the cumulative dose toxicity. Re-irradiation can be given by external beam RT, stereotactic radiotherapy or brachytherapy. The use of brachytherapy and surgery has generally resulted in better outcomes compared to external re-irradiation for the small recurrent leasions[92,93]. Stereotactic radiotherapy, when used for the management of limited residual or recurrent tumor, is associated with a 2 year local tumor control rate of 72%[100].

Photodynamic therapy is a relatively new modality for treating NPC, with good results for local failures of NPC. PDT is an established non-invasive treatment modality for incurable head and neck cancer[101,102]. A photosensitizer is administered to the patient followed by illumination of the tumor with a specific wavelength. This causes tumor destruction. Several clinical trials with first generation haematoporphorin-derived photosensitizers (HpD or Photofrin) have shown that PDT is effective in destroying NPC, with good local tumor control and complete responses in the majority of patients with limited recurrent or persistent disease, while achieving long-term palliation in cases with extensive recurrence[103–105]. Although these results were encouraging, PDT for NPC has not yet been considered as a breakthrough in this field. The two major drawbacks of these studies were (1) the light delivery and (2) the selection of photosensitizer. First, light delivery in the nasopharynx is extremely difficult[106]. It is almost impossible to illuminate the whole tumor area with a lens fiber, guided with

endoscopes or trans-orally with a mirror system. The problem of proper illumination of the nasopharyngeal cavity has now been solved by the development of a special applicator, which allows one-stage illumination of the entire nasopharynx. The second drawback is the use of HpD/Photophrin (the first generation photosensitizer). This photosensitizer has a limited depth penetration of <5 mm and a prolonged light hypersensitivity of several months. Temoporfin (Foscan®), a second-generation photosensitizer and approved in Europe for treatment of incurable head and neck cancer, has a depth penetration of 1 cm and light hypersensitivity of only a few weeks. Research in NPC cell lines by Yow et al confirmed that Temoporfin showed much better PDT efficiency as compared with HpD. These authors also observed significant photo destruction of the mitochondria, especially in Foscan® mediated PDT. Mitochondria are an important sub-cellular target and may play a role in cell death. Temoporfin, in combination with the special designed nasopharyngeal applicator for proper illumination, has the potential to effectively treat recurrent and or residual NPC also in the current Indonesian medical system[103–105].

Distant metastases

The frequency of distant metastasis is 4,4% to 7% at diagnosis and 20% to 27% during follow up[50,107,108]. In case of metastatic NPC (stage IVC) only palliative treatment remains. In chemo-naïve patients, platinum-based regimens are the first choice and give the best results[109]. Chen et al showed the benefit of a combination chemotherapy with radiation for loco-regional disease in case of distant metastases at diagnoses[110]. When the above mentioned strategies have failed, limited options are left. Best response rates for palliative chemotherapy only were found with gemcitabine, capecitabine or docetaxel, with a median survival of 9.5-15 months[111].

Apart from the poor outcome, combination chemotherapy in metastatic NPC in general results in increased toxicity[112].

Targeted therapy

Epidermal growth factor receptor (EGFR) is highly expressed in NPC. A strong expression is associated with poor survival outcome[113]. Combination of the monoclonal antibody against EGFR, cetuximab, with carboplatin in patients with metastatic NPC who have failed prior platinum-based therapies achieved a response rate of 12% and a clinical benefit rate of 60%[69]. Cetuximab has been combined with cisplatin and IMRT in locoregionally advance NPC, demonstrating good tolerability despite a significant incidence of radiation dermatitis,

mucositis and dysphagia[114]. The approach of adding EGFR-targeted therapy to conventional treatment approaches is being actively studied in locoregionally advanced NPC.

Overexpression of the markers associated with hypoxia, including hypoxia-inducible factor 1 Alpha (HIF-1A), carbonic anhydrase 9 (CA-9) and vascular endothelial growth factor (VEGF), is associated with poorer survival outcome in NPC[115]. VEGF and VEGF Receptor targeted therapies, including use of Bevacizumab (Avastin) and small molecule or DNAzyme inhibitors[116], are underway to limit neovascularization in NPC.

In undifferentiated NPC, EGFR overexpression was up to 83% and was found to correlate with primary tumor stages of disease and locally aggressive diseases[117].

Overexpression of epidermal growth factor receptor (EGFR) has been correlated with alterations in cell cycle progression, increased invasive capacity, enhanced angiogenesis, and decreased apoptosis of tumor cells. Overexpression was also associated with larger and more advanced tumor stage and poor prognosis, while EGFR activation was associated with resistance to radiation[118,119]. Study in five NPC cases has found the effectiveness of EGFR blockade in tumors, without the deleterious skin toxicity (skin rash) as commonly found in treatment with other EGFR inhibitors[120].

The viral antigens expressed by the tumor cells are attractive targets for immunotherapy and may be a potential avenue for the development of new therapies for the treatment of NPC[121,122].

When cytotoxic T-lymphocytes (CTL) are added, the EGFR concentration in the tumor cell membranes (EGFRs) is suppressed and the activity of EGFR declines during CTL immunotherapy, which is consistent with the finding of Yuan et al[123,124].

The combination of nimotuzumab (anti-EGFR) and radiotherapy in head and neck cancer is well tolerated and can enhance tumor radiocurability. The addition of nimotuzumab to standard modalities might increase the response and survival rates without significantly potentiating toxicity. A recent study showed combination of nimotuzumab and radiation achieved a highest tumor volume reduction of 98%, and drastic reduction of more than 90% in nodal volume[114].

1

EPSTEIN-BARR VIRUS AND NPC

Introduction to EBV

EBV is the first discovered human tumor virus and is associated with variety lymphomas and carcinomas. In these tumors EBV is actively present in all tumor cells resulting in an increased proliferation and decreased apoptosis. More than 95% of adult population throughout the world is EBV positive as defined by serology. However presence and active role of EBV in NPC pathogenesis can be detected by a rise of IgA titers to EBV antigens. The aberrant serology correlates with tumor development, remission and recurrences[125–127]. Viral DNA load in plasma has been shown as promising alternative marker[128–132], although EBV DNA in whole blood samples does not appear to have similar diagnostic value[133]. Furthermore, viral DNA load in nasopharyngeal (NP) brushings is a direct reflection of aberrant local viral NPC activity in the nasopharynx[134–136]. Besides EBV DNA load in NP-brushings, altered methylation of tumor suppressor gene promoter regions is indicative of tumor presence in situ [137–139]. Therefore, the presence of aberrant antibody responses to EBV and viral presence and activity in all tumor cells may enable us to use the virus and virus-induced changes as biomarker(s) for early detection of NPC, monitoring of therapy outcome/efficacy and prediction of recurrences or distant metastases. The studies presented in this thesis were planned in part to evaluate viral biomarkers contributing to the clinical decision making for patients with NPC.

Epstein-Barr virus

The Epstein-Barr virus (EBV) was discovered 50 years ago by electron microscopy examination of cells cultured from Burkitt's lymphoma tissue by Epstein, Achong and Barr[140]. Four years later, EBV was shown to be the etiologic agent of heterophile-positive infectious mononucleosis[141]. Aberrant anti-EBV antibody responses in NPC patients were a first hint for EBV involvement in NPC[142,143] and EBV DNA was detected in the tumor cells in tissues from patients with NPC in 1970[144]. In the 1980s and 1990s, EBV was shown to be associated with B-cell non-Hodgkin's lymphoma and so-called lymphoproliferative disease (PTLD) in transplant recipients receiving immunosuppressive medication as well as with brain lymphomas and oral hairy leukoplakia in patients with the acquired immunodeficiency syndrome (AIDS)[145–147]. Since then, EBV DNA, RNA and proteins have been detected in tissues from other cancers, including T-/NK-cell lymphomas and Hodgkin's disease and B-cell lymphomas arising in other immune compromised patients[148–151].

EBV is a human herpesvirus (HHV) with a 172 kb long, double-stranded DNA genome that encodes >80 genes[152]. As for other herpesviruses, EBV represents an enveloped virus that consists of a protein core wrapped with DNA surrounded by an icosahedral nucleocapsid and a tegument layer enclosed by a lipid envelope containing glycoprotein spikes for cell attachment. There are 3 main subclasses within the HHV family; alpha-herpesviruses including herpes simplex I and II and Varicella-Zoster virus, beta-herpesviruses including cytomegalovirus (CMV) and HHV6, HHV7 and gamma-herpesviruses including EBV and Kaposi sarcoma herpesvirus (KSHV) or HHV8. EBV is also known as HHV4 and has unique DNA structure and coding sequences separating it from the other HHVs. The gamma herpesviruses can be subdivided into gamma-1 (lymphocryptoviruses, EBV) and gamma-2 (Rhadinoviruses, HHV8). These are the only HHVs directly associated with human tumor formation. Human tumors have been attributed to both human herpesvirus 8 (Kaposi's sarcoma, primary effusion lymphoma and Castleman's disease) and to EBV (Burkitt's lymphoma, various types of classic Hodgkin lymphoma, as well as extranodal NK- and T-cell non-Hodgkin Lymphoma, nasopharyngeal carcinoma, gastric adenocarcinoma, immunodeficiency associated B cell Lymphoma and most recently B cell non-Hodgkin's lymphomas in elderly[153,154]. Humans serve as the only natural host for EBV with target cells predominantly being B cells and epithelial cells[155].

Biology of EBV

EBV infects nearly all humans (>90%) by the time they reach adulthood, but infection occurs mostly at early age[142,156]. EBV can infect a number of different cell types, including B cells and epithelial cells. Under certain condition, it may infect T cells, natural killer cells, monocytes, and smooth muscle cells as well. The mechanisms for entering these cells are different.

EBV enters B lymphocytes by binding with its envelope gp350/220 protein to the CD21 receptor (also known as CR2), which is located at the surface of B cells, whereas viral gp42 interacts with cellular MHC class II molecules as co-receptor. This triggers fusion of the viral envelope with the cell membrane, allowing EBV to enter the B cell[155,157–159]. Entry into epithelial cells is considered to involve cell contact from EBV producing plasma B cells and epithelia, or binding of virions to integrin beta-1 via the envelope BMRF2 protein containing RDG motif and subsequent membrane fusion via gH/gL and gB glycoprotein interaction with other integrins[155,160,161]. Indirect transfer of the virion via monocytes has been described as well as transfer of IgA coated virions via IgA receptors on polarized epithelial cells as alternative routes[162,163].

During acute infection, EBV primarily infects B lymphocytes in the sinonasal lymphoid tissue and replicates in the stratified squamous epithelium of the oropharynx[155,164,165]. The infected individual remains a lifelong carrier of the virus and EBV establishes persistent infection in the host, residing in a small fraction of memory B lymphocytes[166].

Primary infection with EBV typically occurs within the first few years of life, usually asymptomatic and ranges from a mild self-limiting disease in children to infectious mononucleosis (IM) in adolescents and adults in more developed areas. EBV is transmitted by salivary exchange (e.g. pre-chewing food, kissing, etc.), but not every person will get IM symptoms after contact with an EBV carrying individual. EBV infection will result in transient viremia followed by a rapid immune response that will control EBV for life in a permanent well balanced dynamic equilibrium between virus reactivation and immunological control[167,168]. EBV will remain in a dormant state in most humans for long time without serious consequences.

This persistent infection with EBV is reflecting the virus lifelong balance with its human host and where it hides from the immune system via latent infection of B lymphocytes. The latency state allows the virus to be maintained in cells with a highly restricted viral gene expression, which is needed for survival without being recognized and eliminated. The expressed essential viral latency proteins are low immunogenic and have properties to evade the immune system [169–171].

Although EBV exists in 95% of the world population without symptoms and EBV has a strong capacity to immortalize infected host cells, strong host immune responses prevent outgrowth of such potentially dangerous cells. However, in a minority of infected individuals EBV is linked to the development of a variety of lymphoid and epithelial malignancies. EBV is biologically active in the malignant cells of these tumors and each type of tumor has a distinct pattern of EBV gene expression. The EBV genome within the tumor cells shows different patterns (latent) gene expression characteristics for each tumor and reflecting defined episodes (stages) of normal viral activity, as will be described here below.

The life cycle of Epstein-Barr virus

General features

EBV infection of lymphocytes leads to two alternate outcomes. Firstly, EBV can infect naive B cells and let them grow out into memory B cells, which persist with the virus in long-term latency expressing only few of its genes. On the other hand, these B cells can differentiate upon antigen stimulation toward plasma cells that then can produce new infectious virions

and will die. Thus, the EBV infection has two different phases, a latent persistent and lytic reproductive phase[167]. Lifelong infection of the human host relies on this dual phase of infection. Viral replication is naturally enriched in the oral mucosa where memory B cells are routinely stimulated to differentiate after exposure to foreign antigens. The role of mucosal epithelial cells in viral persistence and reactivation is still under debate[172,173].

EBV infection (primary infection and persistence)

Primary EBV infection begins in the oral cavity and affects epithelial cells and naïve B cells. The Waldeyer ring's lympho-epithelial region in nasopharynx and oropharynx, is considered as the location for primary EBV infection, viral replication and EBV persistence. EBV genome will transport into naïve B-cell nuclei followed by B cell immortalization and creation of a B cell memory niche through epigenetic modulation of the host cell. Activation of B cell growth program by EBV gene products will drive proliferation of blasting B cells, at the same time countered and controlled/eliminated by T cells primed by EBV antigens on B cells, acting themselves as the antigen-presenting cells. In the blood, memory B cells with largely quiescent viral genomes will circulate and occasionally enter lymphoid tissues in the head & neck region, to be triggered into activation as plasma cells and switching-on lytic replication. This results in transfer of virus to susceptible epithelial cells further amplify virus production and shed virus into the saliva.

The oropharynx is rich in aggregates of lymphoid tissue, such as the lingual, palatine and pharyngeal tonsils (Waldeyer's Ring). During primary infection, the virus can infect B cells within the tonsillar crypts (squamous epithelium covering tonsils which dips into the connective tissue beneath), thereby entering the "growth program", expanding the number of virus infected cells before entering the latency program[156,165] (Fig. 6). EBV may also persist and replicate in oropharyngeal epithelial cells[172,174]. EBV latent infection of B lymphocytes is necessary for virus persistence, subsequent infectious replication in epithelial cells, and release of infectious virus into saliva. EBV replication in latently infected B cells needs additional triggering by chemical agents or immunoglobulins receptors[175,176].

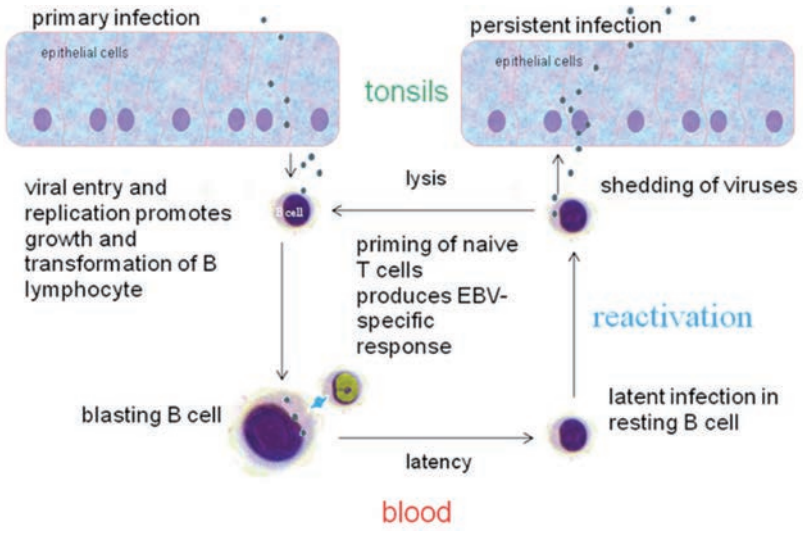

Figure 6 | A Schematic presentation of EBV infection in healthy carriers (adopted from Udumade et al.[142]).

Replicative cycle

The virus is periodically replicated in most asymptomatic carriers of EBV and infectious virions can be recovered in oral secretions[173]. EBV lytic replication will produce infectious virus in mucosal epithelia, after being activated in circulating memory B cells that have re-entered the oropharyngeal lymphoid tissue and upon triggering by antigen or other (chemical or hormonal) triggers that drive B cells to differentiate into plasma cells. This triggering ultimately results in expression of more than 80 viral proteins (figure 7)[175].

Lytic EBV replication is rarely associated with disease except in oral hairy leukoplakia and chronic active EBV syndrome[177,178] and more recently in chronic periodontitis[179].

While EBV latency is dominated by few genes driving cell growth and survival while maintaining the viral genome as a mini-chomosome, the lytic cycle, or productive phase of EBV infection, involves the expression of up to 80 genes and results in the production of new infectious virions. EBV can undergo lytic replication in both B cells and epithelial cells. In B cells, lytic replication normally only takes place after reactivation from latency. In epithelial cells, lytic replication often directly follows viral entry[175]. Virus-encoded DNA polymerase and accessory proteins are required for linear viral DNA replication during the lytic phase of the viral life cycle. This contrasts with latency, when host DNA polymerase copies the viral genome[175].

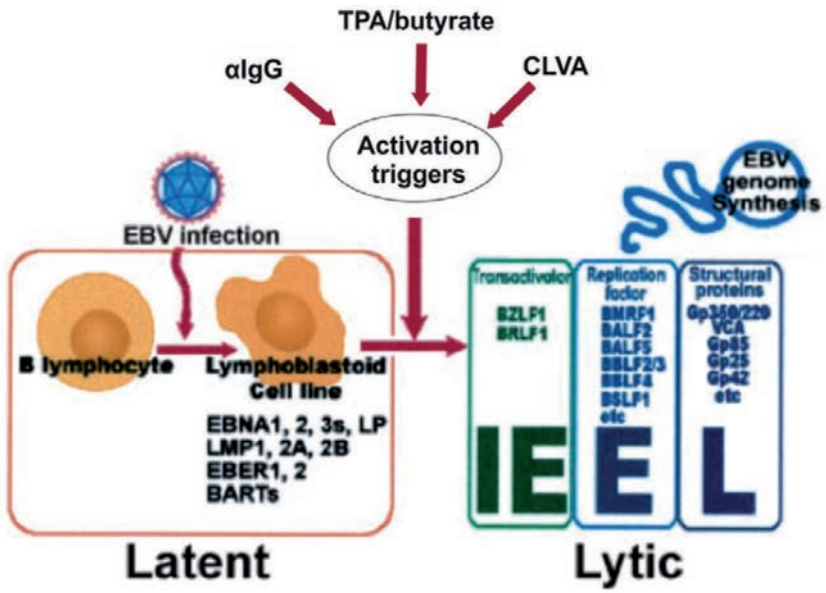

Figure 7 | Life cycle of the Epstein-Barr virus (Adapted from Tsurumi[180]).

Lytic gene products are produced in three consecutive stages: immediate-early, early, and late (figure 7). Immediate-early lytic gene products act as transactivators, enhancing the expression of lytic viral genes as well as host genes. Immediate-early (IE) lytic gene products include BZLF1 (also known as EB1, Zta or ZEBRA) and BRLF1 (Rta or EB2) are part of the IE products that act as transcriptional activators of multiple (methylated) virus and host genes in an auto-stimulatory fashion[180–182].Early lytic gene products have many more functions, preparing the cell for viral DNA replication, enhancing nucleotide metabolism, and blockade of antigen processing. Early lytic gene products include BMRF1, BALF2 and BALF5 that have functions for replication, BGLF5, BNLF2a, and BILF1 that, function in RNA metabolism and blockage of antigen processing. Late lytic gene products tend to code for structural proteins, like viral capsid antigens (VCA p18, p40, p160, encoded in BFRF3, BDRF1 and BcLF1, respectively), which form the viral capsid, membrane antigens (MA gp350/220, gp125/110 encoded in BLLF1 and BALF4) that form the viral envelope and gene products for apoptosis resistance, like BHRF1 and BALF1, and immune evasion like BCRF1, the viral IL-10 homologue[142].

The gp350/220 envelope protein binds to CD21, to mediate EBV attachment to B cells[183,184]. Besides the VCA capsid proteins, the gp125/110 (BALF4), or gB homologue, is believed to be the major immunogen of the VCA complex[185]. Gp350/220 is a target for neutralizing antibodies, but also a prominent constituent of the plasma membrane of the EBV-producing cell and can serve as a target antigen for EBV specific ADCC[186].

Unlike lytic replication for many other viruses, EBV lytic replication does not inevitably lead to lysis of the host cell because EBV virions are produced by budding from the infected cell.

Latency phase of EBV

Three different programs of latent viral gene expressions are defined (Table 2) and can be observed in defined EBV-linked disease entities, as well as certain cell lines in vitro.

Type I latency is characterized by a limited spectrum of latent viral gene expression, namely non-coding EBER and BART transcripts along with EBNA1 protein. This pattern is found in circulating memory B lymphocytes of healthy viral carriers. Burkitt's lymphoma is an EBV-related tumor characterized by latency type I gene expression.

Type II latency is mostly seen in tumors arising in **immunocompetent hosts** characterized by EBNA1, LMP1 and LMP2 protein expression in addition to the presence of non-coding EBER and BART transcripts, as seen in Hodgkin's disease, T cell Lymphoma, and NPC, with the latter also expressing the BARF1 protein. EBV associated gastric carcinoma (GC) shows similar type-II latent gene expression with BARF1 but without LMP1.

Type III latency is mostly seen in B cell lymphoproliferative diseases and lymphoma **immunocompromised hosts** and refers to the full spectrum of latent viral gene expression as found transiently in acute infectious mononucleosis[187].

The higher EBNA proteins (EBNA2, 3a, 3b, 3c, 5) are highly immunogenic and therefore this latency pattern is only observed in the absence of an adequate T cell response as in immunocompromised hosts[168]. In immunocompromised individuals, EBV is related to post-transplant (PTLD) and AIDS-related lymphoproliferative disorders, autoimmune lymphomas and lymphomas in the elderly, but not particularly with NPC[154,187,188]. In HIV carriers Hodgkin's disease is virtually always EBV positive as it is in most developing countries[189].

Table 2 | Expression of EBV latent genes in disease (adopted from Cohen[190]).

Pattern of Latency	EBNA1	EBNA2	EBNA3	LMP1	LMP2	EBERs	BARTs	DISEASE
Type 1	+	-	-	-	-	+	+	Burkitt's lymphoma,
Type 2	+	-	-	+	+	+	+	**NPC**, GC, Hodgkin's disease, peripheral T-cell lymphoma
Type 3	+	+	+	+	+	+	+	PTLD, X-linked LPD , infectious mononucleosis
Other	+/_	-	-	-	+/-	+	+	Healthy carrier

Function(s) of EBV latent genes

Latent infection in benign and malignant cells is characterized by limited expression of viral proteins to avoid immune recognition and destruction. Most latent protein functions have been defined in the background of EBV-infected and immortalized B-cells as cultured in vitro. The resulting lymphoblastoid cell lines (LCLs) express most latent EBV gene products**,** like six nuclear proteins (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP), three membrane proteins (LMP-1, LMP-2A, LMP-2B), EBV-encoded small RNAs (EBER1 and EBER2) and rightward transcripts from the BamHI A region (BARTs) (table 3).

Table 3 | Function of EBV latent genes

Gene products	Function
EBNA1	Maintenance of EBV episome in dividing cells through sequence-specific binding at OriP and linking EBV genome to chromosomes. Inhibits proteosomal degradation and presentation to MHC class I via gly-ala repeat. Destabilises p53 via interaction with cellullar ubiquitin-specific protease (USP7) and interferes with PML-bodies affecting DNA damage repair. Enhances cellular gene transcription and induces surviving expression.
EBNA2	EBV transcriptional enhancer and oncogene required for B cell transformation together with EBNA-LP (and LMP1). Interact with RBP-Jκ to transcriptionally activate cMyc, Runx-5, CD23 and other cellular genes as well as viral LMP1 and LMP2 early after infection.
EBNA-LP	Upregulation of transcription factors needed for B cell transformation and growth.
EBNA-3A	Essential for EBV-mediated transformation of primary B lymphocytes and interacts with RBP-Jκ, balancing EBNA2 binding to RBP-Jκ transcription factor. Together with EBNA3C prevents pro-apoptotic Bim expression in Burkitt cells with enhanced c-Myc expression, stimulating survival.
EBNA-3B	Transcriptional regulator, not essential for initial transformation
EBNA-3C	Essential for EBV-mediated transformation of primary B lymphocytes and interacts with RBP-Jκ. Promotes LMP1 expression in the presence of EBNA2.
LMP1	Essential for EBV transformation of B cell in vitro and drives proliferation through NF-κB, AP-1 and JAK/STAT pathway activation. Mimics CD40 activity by providing growth and differentiation signals to B cell. Up-regulation of anti-apoptotic proteins (Bcl-2, A20). Induces malignant transformation in human cells and transgenic mice. Activates transcription of epidermal growth factor receptor (EGFR) in epithelial cells.
LMP2A	Inhibits signaling through BCR and promotes the proliferation and survival of B cells. Promotes migration and stem-cell characteristics in epithelial cells.
LMP2B	Modulator of LMP2A
EBER1,2	Counteracts the anti-viral effects of interferon and PKR activation in infected B cells. Induces TLR3 signaling in external recipient cells, stimulating innate immunity.
BARF1	Induce tumorigenicity and malignant transformation in primary epithelial cells. Induces apoptosis-resistance via up-regulating Bcl-2. Share homology with c-Kit, binds CSF1 and modulates monocytes activation.
BARTs	Encode >40 miRNAs that regulate EBV latent infection and modulate host immune responses by targeting a variety of viral and cellular messenger RNAs.

Epstein-Barr nuclear antigen 1 (EBNA1)

EBNA1 is a DNA-binding nuclear phosphoprotein which is required for the replication and maintenance of the episomal EBV genome in dividing cells through binding to the origin of plasmid replication (Ori-P) on the viral genome[191]. The tethering of the viral genome to the host chromosome is mediated via so-called AT-hook domains within GR-repeats of EBNA1, allows the replication of EBV during cell division by the replication machinery of the host[192]. EBNA1 also functions as a transcriptional enhancer, driving defined host promoters into altered activity[193–195] and induces Survivin expression leading to apoptosis resistance[196]. EBNA1 is the only EBV protein that is expressed in all latently EBV-infected cells. Although EBNA1 is a foreign protein to the host, EBV-infected cells expressing EBNA1 are not killed by CTLs. This is due to a inhibitory effect of the protein's Gly-Ala repeat on proteasomal processing thereby preventing endogenous MHC class I-restricted presentation[197,198]. EBNA1 is capable of inducing genomic instability and interferes with p53 stability and DNA repair mechanisms, enhancing the risk for genomic alterations in the host cell, thus increasing the potential for cancer development[199–201].

Latent membrane protein 1 (LMP1)

LMP1 is considered the major EBV oncogene and functions as a viral mimic of the TNFR family member CD40. LMP1 signals constitutively without the need for a ligand and thereby exhibits properties of a classical oncoprotein, inducing promotion of cell growth and inhibition of apoptosis in a variety of cell types in vitro[152]. The regulation of LMP1 expression occurs both at the transcriptional and post-translational level and LMP1 is secreted into exosomes[202]. LMP1 is often present in NPC and can be detected in pre-invasive lesions of nasopharynx[203]. LMP1 is considered to, have an important role in the pathogenesis NPC and its detectable expression showed to correlate with poor prognosis[204]. LMP1 induces expression of the epidermal growth factor receptor (EGFR) in epithelial cells and EGFR is expressed at high level in NPC[205]. The induction of EGFR expression may be an important contributing factor to the deregulated cellular growth in this epithelial tumor.

LMP1 induces secretion of IL-6 in epithelial cells and decreases expression of cytokeratins and E-cadherin[206]. LMP1 inhibits apoptosis in B-lymphocytes triggering expression of the Bcl-2[207]. LMP1 achieves its wide-ranging phenotypic effects through the activation of multiple signaling cascades. Its activates the NF-κB, JNK and JAK/STAT pathways through direct interaction with pathway intermediary proteins[208,209]. LMP1 induces expression and secretion of MMPs thereby promoting metastatic behaviour, and induces cytokine secretion thereby providing growth stimuli (IL-6), neo-angiogenesis (IL-8) and immunosuppression

(IL-10). Secreted in exosomes, LMP1 may have modulatory functions in recipient cells in the tumor cell microenvironment and mediate immune evasion[171,210–212].

Latent membrane protein 2a and 2b (LMP2a and -2b)

EBV LMP2A prevents reactivation of EBV from latently infected cells by blocking B-cell receptor tyrosine kinase phosphorylation[205]. LMP2 is not required for B cell transformation, but essential for long term persistence for the viral episome by providing B cell survival signals in the lymphoid organs [150,213]. LMP2b modulates the function of LMP2a in regulating BCR signaling, driving the latently infected B cells to lytic reactivation[150]. In epithelial cells LMP2a is considered to contribute to migration and invasion and induce stem-cell like characteristics[214–216]. LMP2a is considered to cooperate with LMP1 in epithelial transformation and carcinogenesis[217,218].

BamHI rightward frame 1 (BARF1) protein

The EBV-encoded BARF1 gene is located in the BamHI-A fragment of the EBV genome and has oncogenic activity, encodes 221 amino acids[219]. BARF1 functions as a viral oncogene, immortalizing and transforming epithelial cells of different origin by acting as a mitogenic growth factor, inducing Cyclin-D1 expression, and up-regulating anti-apoptotic Bcl-2, stimulating host cell growth and survival[220]. Since BARF1 is expressed in tissues of various EBV-associated epitheloid malignancies, the possibility cannot be excluded that BARF1 expression in EBV-associated epitheloid malignancies reflects spontaneous induction of the lytic cycle in carcinoma cells. Because expression of the BARF1 gene is induced on the induction of the lytic cycle in EBV-positive cell lines[221,222] and in Burkitt's lymphoma cell lines, expression of BARF1 in NPC tissues is thought to reflect spontaneous induction of the lytic cycle in carcinoma cells. Quantitative real-time RT-PCR assay revealed that BARF1 was highly expressed in nasopharyngeal carcinoma (NPC) and EBV-positive gastric carcinoma tissues. In the absence of expression of lytic gene BARF1 is expressed in NPC and EBV-positive gastric carcinoma tissues as a latent gene and this suggests that BARF1 plays a role in the pathogenesis of these malignancies[135,223,224]. BARF1, an intracellular and secreted protein, has multiple pathogenic functions and also can function as a target for immune responses[220].

EBV-encoded small RNAs (EBERs)

The EBV encoded small RNAs (EBERs) comprise the highly abundant EBER1 and EBER2 molecules of 162-176 nucleotides in size. EBER transcripts are expressed in all latency states and represent a reliable target for detecting and localizing EBV in tissue sections by RNA in

situ hybridization[225]. The EBERs are expressed in many of the malignancies linked to EBV, including NPC and presumably contribute in some way to the maintenance of latency in vivo[226].

The high level of sequence conservation, suggests that EBERs are important in EBV biology, even though EBERs do not play a role in establishment of latent viral infection and replication[227]. EBERs were found to interact with cellular proteins that play a key role in antiviral innate immunity. EBERs can be secreted from EBV-infected cells in exosomes and protein-RNA complexes and are recognized by toll-like receptor (TLR) 3, leading to induction of type-I IFNs and inflammatory cytokines, and subsequent immune activation[228]. Furthermore, EBER1 was detected in sera of patients with active EBV disease, suggesting that activation of TLR3 signaling by EBER1 can account for some pathogenic characteristics of reactive EBV diseases[229].

EBER in situ hybridization is the gold standard for detecting and localizing latent EBV infected cells in tissue samples in every benign and malignant lesion[63]. EBER in situ hybridization is often helpful in making the correct diagnosis[230,231]. In cases of metastatic cancer of unknown origin, it is reasonable to consider NPC if EBV is present in the tumor cells[232].

Rightward transcripts of the BamHI-A region of the viral genome (BARTs)

BARTs are abundant transcripts derived from the BamHI-A fragment of the viral genome and expressed in all EBV infected cells and have elevated levels in NPC and GC. Structural analysis of the BARTs revealed the presence of several open reading frames. These are RPMI-1 and -2, A73 and BARF0 or depending on the splicing of the transcript RK-BARF0[233,234]. However the protein products of these putative genes remain undefined[235]. The function of most of the BARTs is still under investigation, but their detection in infected B cells and in many EBV-associated malignancies suggests that they might have an important role in viral persistence and pathogenesis[150]. Recently BARTs were found to encode for about 40 individual microRNA species, that are abundantly expressed in NPC and other EBV-driven cancers[236–238]. MicroRNAs (miRNAs) are small non-coding RNAs that play important roles in post-transcriptional gene regulation. In animal cells, miRNAs regulate their targets by translational inhibition and mRNA destabilization[239]. Recent findings show that miRNAs can also modulate the cell microenvironment, enabling immune escape and metastasis[240,241].

EBV is linked to NPC carcinogenesis

EBV related malignancies primarily arise from infected lymphocytes and epithelial cells, leading to lymphomas and carcinomas, respectively. The tumors are latently infected with

EBV yet express distinct subsets of viral proteins that are contributing to growth, survival and immune evasion. In some of the viral cancers, viral proteins are barely expressed, but the viral small and miRNAs can alter growth by decreasing expression of negative regulators of cell growth such as tumor suppressors and cellular proteins that induce apoptosis[242] (Fig. 8).

In Southern China NPC is the third most common malignancy among men, where the incidence is approximately of 30–80/100,000 in the Cantonese region around Guangzhou, Province of Southern China[9,243]. Genetic as well as environmental factors play a role in the cause of the disease[1]. The disease is classified by World Health Organization (WHO) into three histological types: I, squamous; II, non-keratinizing; III, undifferentiated[244]. Circular EBV is present in NPCs with a latency type II. High titers of serum IgA to EBV viral VCA and EA antigens have diagnostic value. Rise in IgA titers may be evident several years before the development of an undifferentiated NPC[126]. The link between EBV and NPC was initially based on serological findings[143,245], and later confirmed by detection of viral DNA, RNA and protein in the tumor cells in situ using nucleotide probes and specific antibody reagents[144,246,247]. High titer antibodies to VCA and early antigen especially of IgA class, or high titers that persist after therapy, where found to be associated with a poorer prognosis[248]. Detection of EBV DNA in peripheral blood plasma is an important risk factor that indicates a significantly high likelihood of developing distant metastasis as well as poor survival[249,250].

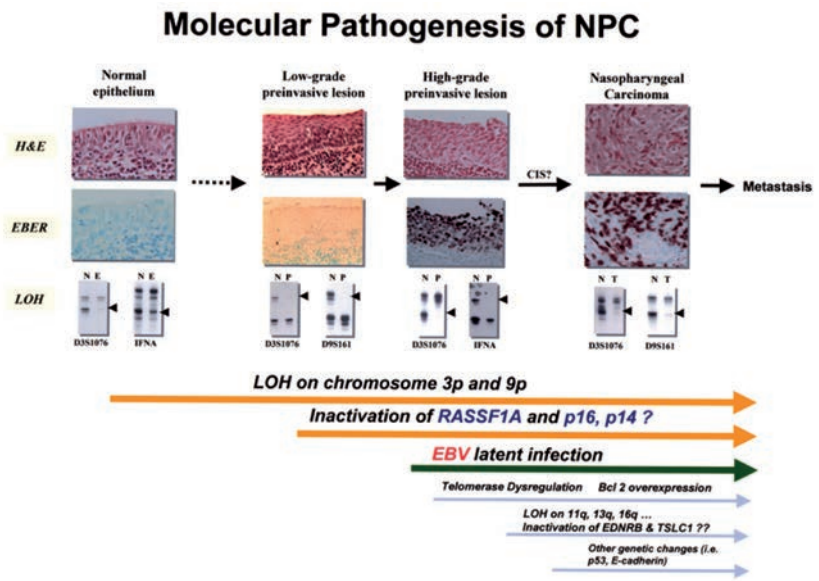

Figure 8 | Role of EBV in the pathogenesis of NPC (Adopted from Young LS and Rickinson AB[251], Lo KW et al.[252]).

The pathogenesis of NPC involves 3 factors. First, early and repeated EBV infection and chronic triggering of aberrant viral replication by exposure to environmental factors like dietary components such as nitrosamines in salty fish; Secondly, loss of heterozygosity (LOH), usually occurring early in the NPC pathogenesis and possible induced by EBNA1 expression as outlined above, will produce low-grade pre-invasive lesions. Thirdly, accumulation of epigenetic and genetic lesions in key regulatory genes leading to loss of cell cycle control and enhanced survival[138].

In EBV positive NPC and GC, the tumor cells carry monoclonal viral genomes, which indicates that EBV infection must have occurred prior to expansion of the malignant cell clone[253]. EBV infection has been detected both by EBER RNA in situ hybridization and by the presence of monoclonal EBV genomes in high-grade pre-invasive lesions (severe dysplasia and carcinoma in situ) in the nasopharynx, but not in low-grade disease[203]. Multiple genetic changes have been found in NPC, with frequent deletion of regions on chromosomes 3p, 9p, 11q and 14q and promoter hypermethylation of specific genes on chromosomes 3p (RASSFIA and retinoic-acid receptor B2) and 9p. Deletions of some chromosomal regions of low-grade dysplastic lesions from high-risk persons in the absence of EBV infection in those lesions, indicate that genetic defects may occur earlier than EBV infection. Therefore in the pathogenesis the genetic events caused by exposure to chemical carcinogens might cause predisposition to subsequent EBV infection leading to NPC[254]. Interestingly, recent data indicate that the EBV genome is more stably maintained in epithelial cells aberrantly expressing Cyclin D1, which may be an initiating event in the pathogenesis of NPC[255].

Immune response to EBV and evasion of the immune system by the virus

Infection of humans with EBV results in both lifelong humoral and cellular immunity to the virus. Although the finding of antibodies directed against virus structural proteins and the EBNAs is important for the diagnosis of infection, the cellular immune response is more important for the control of EBV infection. Natural killer cells and CD4+ and CD8+ cytotoxic T cells control proliferating EBV-infected B cells during primary infection[168]. After recovery from acute infection, HLA-restricted cytotoxic T cells are important in controlling EBV, and CD8+ T cells are targeted to both replicative and latent antigens. Many of the cytotoxic T cell responses directed against latent proteins are targeted to the EBNA3 proteins controlling the potentially dangerous latency-III stage of infection[168,256] .

The ability of EBV to persist, despite potent immune effector responses against it, indicates that the virus has evolved strategies to elude the immune system. EBV encodes a cytokine and a cytokine receptor that may be important for modulating the immune system to allow

persistent infection. The BCRF1 protein mimics the activity of interleukin-10 by inhibiting interferon-g synthesis by human peripheral-blood mononuclear cells in vitro[257]. The EBV BARF1 protein functions as a soluble receptor for Colony-Stimulating Factor 1. Since Colony-Stimulating Factor 1 normally enhances the expression of Interferon-g by monocytes, BARF1 protein may function as a decoy receptor or scavenger to block the action of the cytokine[220,258]. Since interferon-γ and Interferon-α inhibit the outgrowth of EBV-infected cells in vitro, the BCRF1 and BARF1 proteins may help the virus to evade the host's immune system during acute EBV infection or reactivation of virus from latently infected cells.

EBNA1 has been shown to block its own degradation by proteasomes in the cell[197]. Since viral proteins are normally broken down by proteasomes to peptides for presentation to cytotoxic T cells, the ability of EBNA-1 to inhibit its degradation may allow the protein to avoid triggering the activation of cytotoxic T cells. In addition, lytic phase proteins encoded in BGLF5, BILF1 and BNLF2a genes interfere with antigen processing and presentation in MHC-I avoiding elimination of cells that switch to virus production[259–261].

EBV encodes at least two proteins that inhibit apoptosis. The EBV BHFR1 protein, expressed early during lytic reactivation and some stages of viral latency is a homologue of the human Bcl-2 protein, which also blocks apoptosis[262], whereas EBV LMP1 up regulates the expression of several cellular proteins that inhibit apoptosis, including Bcl-2 and A20[263]. In addition, LMP1 contains an immunosuppressive domain, which –upon LMP1 secretion-, can interfere with T-cell activation[171,210]. In vivo evidence for local evasion of cellular immune responses by NPC tumor cells is illustrated by induction of silencing Treg cells in the tumor, not present in the circulation[264]. Together these data indicate that, although NPC tumor cells express potentially immunogenic viral proteins, the tumor cells exhibit several ways of evading these responses, resulting in extended survival and continued growth. However, raising the level and activity of anti-EBV immunity by vaccination or immunotherapy, may provide future options for intervention[265].

Humoral responses to EBV antigens are broad and strongly elevated, in both IgG and IgA classes. Predominant antigens recognized by these antibodies include the latent EBNA1 protein as well as EA, VCA antigens[266]. High level neutralizing antibodies exist in NPC patients[267]. It is considered that the humoral immune response to EBV antigens in NPC is reflecting antigen expression during tumor development, rather than being protective. However, these aberrant anti-EBV antibody responses are very useful in diagnosis of NPC, as outlined below.

EBV related laboratory tests

The fact that EBV is present and active in almost all NPC cases makes EBV an ideal tumour marker for NPC. Quantitative analyses of EBV antibodies and EBV DNA have been shown to be clinically useful for the early detection, monitoring and prognostication of NPC[129,268,269]. Early detection is essential since patients with NPC usually enter the clinic with an advanced stage of disease decreasing therapy success rate. Population screening for high-risk individuals would allow the recognition of early-stage NPC. To aid the clinical diagnosis, early detection can be achieved by new EBV-based serodiagnostic tests and parallel molecular diagnostic tests. The viral markers could also be used for the prediction of recurrence in addition to monitoring therapy responses to guide the clinicians in decision making. A wide variety of diagnostic approaches for prediction, diagnosis and prognosis of EBV-associated diseases have been described

Serodiagnostic approaches

The initial link between EBV and NPC was based on identification of EBV-specific serological abnormalities in NPC patients to viral antigens present in newly discovered Burkitt Lymphoma cells[143]. In particular the presence of IgA antibodies to several EBV antigen complexes defines NPC patients, as confirmed in larger studies by the Henles[245]. For diagnosis of acute versus past EBV infection and definition of EBV carriership in immunocompetent hosts EBV-specific serological testing is the gold standard. EBV-specific serology involves the analysis of antibody responses to distinct viral antigens, originally defined by patterns of immunofluorescent staining on Burkitt-lymphoma cells, comprising the viral capsid antigen complex (VCA), the EBV nuclear antigen complexes (EBNA) and the early antigen complex (EA), the latter divided into diffuse (EAd) and restricted (EAr) complexes, with different serological implication[245]. In recent years the molecular basis of EBV serology has been defined, gradually leading to replacement of the laborious and poorly reproducible immunofluorescence tests to more defined and reliable assays[270–272]. Early serological studies in South-East Asia and Indonesia have shown that at age 5 nearly 100% of Indonesians are infected by EBV[273]. Serological test consists of agglutination tests, immunofluorescence, immunoblot or enzyme immunoassays (EIA) and more recently automated and multiplex tests which allow accurate definition of acute or convalescent EBV infection[274,275].

At the time of acute infection IgM anti-VCA will arise first, followed by IgG anti-VCA and IgG anti-EA, with symptoms of primary infection and a positive heterophile test. After symptoms resolve around 2 months post infection, remote infection is characterized by persistent IgG anti-EBNA and VCA without IgG-EA, reflecting lifelong EBV carriership. IgG-EA

may reappear without symptoms upon viral reactivation or EBV related neoplasia few years late[272] (Figure 9).

For EBV related malignancies serology alone appeared not to be adequate for diagnosis. Although patients with EBV associated tumor have often higher titers of IgG antibodies against EBV proteins, this is not always related to tumor presence and is not specific for malignancy as it can also be found in autoimmune disease, chronic EBV infections and other immune disfunctions[276–278]. In contrast, NPC often associates with elevated levels of IgG-VCA antibodies, particularly against the lytic VCA-p18 protein and the EBNA1 protein, and IgA antibodies against early antigen (EA)[129,266,279–282].

IgA antibodies against EBV antigens in serum of patients with NPC, reflect the tumor's origin in the mucosa of the nasopharynx. The anti-viral capsid antigen IgA antibody (IgA-VCA), measured by indirect immunofluorescence or ELISA, is the most widely used antibody marker for diagnosis and screening. The preferred assay is based on ELISA, since the interpretation of the immunofluorescence assay is subjective and the technique needs expert skills[283]. EBV-IgA testing, combining VCA with EBNA1 or VCA with EA antigens successfully used for defining high risk population of NPC and to evaluate prognosis and detection of recurrences after completion of therapy[266,284–286]. The first mass-screening studies were performed in complete city populations in Southern China by Zeng Yi [280,287], followed by multiple large-scale studies confirming the use of IgA-VCA (and –EA) for NPC diagnosis The sensitivity of EBV-IgA in the diagnosis of WHO type II and II NPC in areas both endemic and non endemic for the disease has been reported to be 85-90% [266,288–291]. The EBV-IgA markers frequently precede the appearance of NPC and may serve for early-stage NPC detection and possibly also serve as markers of remission and relapse [126,248,284,292].

Ji et al confirmed that elevation of the EBV antibody levels preceded the clinical onset of NPC by as much as prior to the clinical onset, and that the antibody level is subsequently elevated and maintained at high levels[126]. Most IgG of healthy EBV carriers recognize a restricted pattern of EBV proteins in immunoblot, which includes strong IgG reactivity against VCA-p18 and EBNA1[270,293]. In many NPC patients IgA antibodies against EBV proteins are detectable, even before the carcinoma becomes clinically evident. These antibodies are reflecting the abnormal activity of EBV within the nasopharynx. Both IgA-VCA and IgA-EAd have been proposed for NPC follow-up monitoring post treatment.

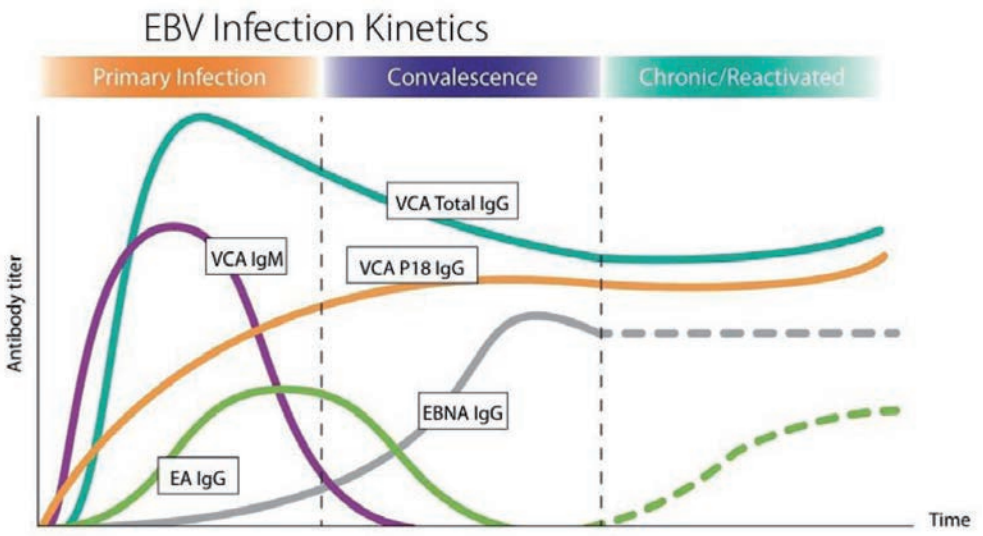

Figure 9 | EBV infection kinetics with permission Cyto-Barr BV, Netherlands

Molecular diagnostic approaches

Next to the indirect serological assays for NPC detection, a direct measurement of NPC presence could be obtained by detecting and quantification of the viral genome within the tumor cells. EBV DNA levels in nasopharyngeal brushings from NPC patients are significantly higher than in brushing from healthy controls. NPC may be more directly reflected by elevated viral DNA levels plus carcinoma-specific viral transcriptional activity at the site of the primary tumor, this hypothesis is analyzed in recent reports, showing elevated EBV DNA loads in NP brush samples in NPC patients using non-standardized PCR techniques[135,136,294].

Patients with active infection or EBV-related cancer tend to have high levels of EBV DNA in the cell-free fraction of blood (plasma or serum), whereas in healthy carriers the virus is restricted to the intracellular compartment of the blood. Quantitative EBV DNA measurement is essential for differentiating the low-level infection of healthy carriers from the high levels characteristic of EBV-related disease[272].

EBV DNA levels begin to rise within 2 weeks after primary infection after which the viral load declines to nearly undetectable levels. In most EBV infected healthy individuals EBV DNA is undetectable in plasma or serum or remains low positive for life. The viral DNA load will increase according to the development of EBV related malignancy, decreases after treatment rapidly, and shows elevated levels again if the tumor relapses. Most clearly this

is seen in transplant recipients developing EBV-driven PTLD, where DNA viral load in whole blood shows, rising levels prior to diagnosis and clinical relapse, and declining levels indicate success of treatment[295–297].

EBV viral load and circulating free EBV DNA are markers of tumor burden in NPC patients where it is useful for diagnosis, prognostication and monitoring of the disease in response to therapy[249,269,298–300]. Plasma EBV DNA appears more sensitive and reliable than whole blood cell EBV DNA for diagnosis staging and therapeutic effect evaluation at a molecular level in NPC clinical practice[133,269]. Levels of plasma EBV DNA may rise before clinical diagnosis, implying that screening in high-risk groups, might be beneficial for early-stage NPC detection[132,193,301–304].

The detection of plasma EBV DNA might directly reflect tumor growth and decline[130,305,306], and has proven to be an important and sensitive index in diagnosing the residual and relapse of NPC[129,301,302]. Successful therapy is marked by a decline to baseline, and rising levels may indicate a relapse[305]. The increased number of EBV DNA copies in blood during the initial phase of radiotherapy suggests that viral DNA was released into the circulation after cell death[193,300]. The plasma EBV DNA load may improve the accuracy of diagnosing NPC in high-risk individuals, but it appears to have limited value in screening patients who have early stage NPC and predicting NPC development[307].

AIM AND OUTLINE OF THE THESIS

The improvement of early diagnosis of NPC should be first priority, since the symptoms related to NPC in the early stage are usually nonspecific, and most NPC patients are currently diagnosed in the advanced stage, resulting in poor prognosis. Patients with a history of unexplained unilateral serous otitis media, frequent nasal bleeding or chronic rhinitis not associating with allergy of bacterial infection, should be carefully examined to rule out NPC[1,47]. Endoscopy plays a key role in detecting the early NPC lesions, and an endoscopic biopsy with pathological examination enables definitive diagnosis. Molecular biomarkers and non-invasive diagnostic sampling methods are under examination as a tool for the detection of early NPC lesions as well as the early diagnosis of recurrent, residual or metastatic NPC lesions. In addition to EBV serological assays, which have been established as primary diagnostic markers, EBV-DNA/ RNA testing coupled to the use of non-invasive nasopharyngeal brushings for early and specific recognition and post-treatment monitoring of NPC may be relevant. This approach, which resembles cervical cancer screening methods, may provide important information on EBV positive NPC specific activity (EBV-DNA load

and BARF1 mRNA expression) at the anatomical site of tumour development, with direct diagnostic and prognostic relevance.

In this thesis study EBV-DNA load will be compared with peptide-based molecular EBV-IgA serology recently developed and evaluated in another Indonesian center. The method for non-invasive diagnosis by measuring EBV-DNA in nasopharyngeal brushings as well as in parallel whole blood samples will be evaluated against other clinical and laboratory markers and compared to the biopsy as golden standard. Today most patients come to the hospital with advanced stages of disease. Our teaching methods and laboratory test results will permit approaches to downstage the disease, monitoring treatment result, thereby allowing the design of future improvements in treatment success and patient survival. Finally we aim to validate photodynamic therapy as simple cost-effective treatment of local tumor mass, either for tumor debulking at intake or as treatment for local recurrent disease.

Chapter 2: The incidence and the etiology of NPC and its geographic prevalence is described in Indonesia, which is still an unexplored region with a considerable NPC incidence. Due to the lack of a national cancer registry a nation-wide overview of NPC incidence and mortality in the Indonesian community is lacking. We evaluated the incidence in our hospital, which is an important referral institute and some data of NPC hospital based repositories at other academic hospitals throughout the country. We find NPC to be the most prevalent head and neck cancer with an overall incidence of about 6/100,000 population and an increasing tendency of NPC occurrence in young adults. This should be explored further to see if juvenile NPC has different epidemiology or biology of EBV

Chapter 3: In this chapter we investigated the primary diagnostic value of EBV-DNA load and mRNA detection in non-invasive nasopharyngeal (NP) brushings, obtained prospectively from consecutive Indonesian ENT patients with suspected NPC and controls from Yogyakarta, Indonesia. Routine NP biopsy was taken for pathological examination and EBER-RISH analysis. NPC patients were found to have extremely high EBV DNA loads compared to the non-NPC controls. It was concluded EBV DNA load measurement combined with detection of BARF1 mRNA in simple NP brushings allows accurate non-invasive NPC diagnosis and can be used in adjunct to serological techniques to confirm NPC presence. The brush related EBV markers reflect carcinoma specific EBV involvement at the anatomical site of tumor development and its use reduces the need for invasive biopsies.

Chapter 4: By using same techniques as chapter 3 the diagnostic value of EBV DNA load in brush, blood and EBV-IgA serology was evaluated at the time of diagnosis and 8 weeks post-treatment in an independent and different population in Indonesia. We confirmed the initial findings described in Chapter 3 and observed that EBV DNA levels in brushings and whole

blood showed a significant reduction at 2 month post-treatment, which was not reflected in EBV-IgA serology. EBV DNA markers may therefore be suitable for treatment monitoring.

Chapter 5: In this chapter we analyzed the longitudinal implementation of non-invasive EBV-DNA load in brushings and whole blood, plus EBV-IgA serology in follow-up monitoring of patients with NPC undergoing chemoradiation therapy. We aimed to evaluate diagnostic methodology for prediction of recurrent disease and studied the dynamics of viral biomarkers in NP brushes and whole blood from patients with NPC during and after treatment. It proved difficult to properly follow-up the patients initially included in this study due to multiple logistic and social reasons. From the available data we conclude that monitoring EBV DNA load decrease in NP-brushings reflects the early phase of treatment and, and can reflect local recurrences. Blood levels of EBV DNA appear less informative because these are frequently low or negative at diagnosis. However in some patients DNA load in blood increased and predicted recurrence. Serology has no value in follow-up due to limited dynamics.

Chapter 6: In this chapter we further analyzed the presentation, treatment and treatment outcome of young patients with NPC. Our treatment results for young patients with NPC are poor compared to literature. These results are caused by advanced stage of disease at presentation, distinct problems in the health care system, social and geographic problems, and other problems causing prolongation of treatment time.

Chapter 7: Since treatment of persistent and recurrent of NPC remains challenge, we evaluated treatment by PDT for these patients. We evaluated safety and efficacy of Temoporfin mediated PDT. We conclude that PDT with a specifically designed NP applicator is a simple technique and can be used for treating local recurrent disease.

Chapter 8: Facing the fact that > 85% patients came with advance NPC disease, we evaluated the knowledge of general practitioners working in primary health care centers by giving questionnaires, lectures and symposia for providing further information on NPC. It is shown that the education increased short-term knowledge and should be followed with a further training program and improving medical connection and interaction between the hospital and the primary health care centers. Improvement in the awareness and knowledge of early signs and symptoms of NPC at the level of physicians and the general population will undoubtedly contribute to the earlier detection of the disease.

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Chapter 2

Nasopharyngeal carcinoma in Indonesia: epidemiology, incidence, signs, and symptoms at presentation

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ABSTRACT

Among all head and neck (H&N) cancers, nasopharyngeal carcinoma (NPC) represents a distinct entity regarding epidemiology, clinical presentation, biological markers, carcinogenic risk factors, and prognostic factors. NPC is endemic in certain regions of the world, especially in Southeast Asia, and has a poor prognosis. In Indonesia, the recorded mean prevalence is 6.2/100 000, with 13 000 yearly new NPC cases, but otherwise little is documented on NPC in Indonesia. Here, we report on a group of 1121 NPC patients diagnosed and treated at Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia between 1996 and 2005. We studied NPC incidence among all H&N cancer cases (*n* =6000) observed in that period, focusing on age and gender distribution, the ethnic background of patients, and the disease etiology. We also analyzed most prevalent signs and symptoms and staging of NPC patients at first presentation. In this study population, NPC was the most frequent H&N cancer (28.4%), with a male-to-female ratio of 2.4, and was endemic in the Javanese population. Interestingly, NPC appeared to affect patients at a relatively young age (20% juvenile cases) without a bimodal age distribution. Mostly, NPC initiated in the fossa of Rosenmuller and spreaded intracranially or locally as a mass in the head. Occasionally, NPC developed at the submucosal level spreading outside the anatomic limits of the nasopharynx. At presentation, NPC associated with hearing problems, serous otitis media, tinnitus, nasal obstruction, anosmia, bleeding, difficulty in swallowing and dysphonia, and even eye symptoms with diplopia and pain. The initial diagnosis is difficult to make because early signs and symptoms of NPC are not specific to the disease. Early-age Epstein-Barr virus (EBV) infection combined with frequent exposure to environmental carcinogenic co-factors is suggested to cause NPC development. Undifferentiated NPC is the most frequent histological type and is closely associated with EBV. Expression of the EBV-encoded latent membrane protein 1(LMP1) oncogene in biopsy material was compared between NPC patients of < 30 years old and those of ≥30 years old, matched for sex and tumor stage. Higher LMP1 expression in patients of <30 years old was observed, which was related to more locoregional progressivity. Increased medical awareness of prevailing early stage signs and symptoms coupled to use of EBV-related diagnostic tumor markers may lead to down-staging and timely treatment to improve survival of patients with this aggressive disease.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a rare malignancy throughout most parts of the world, with a prevalence usually less than 1/100 000[1,2]. NPC is a highly prevalent malignant disease and a leading cause of death in several regions in southern China, and recently improved registry data indicate medium to high prevalence in other countries in Southeast Asia as well. The Guangdong province in South China has the highest prevalence in the world, with approximately 20 to 40 cases per 100 000 inhabitants depending on the region[3-7]. Earlier work showed that Cantonese "boat people" had the highest incidence of NPC (54.7/100 000)[8]. The data from Guangdong were recently paralleled by findings in native Bidayuh people of Serawak, Malaysia, who also had a high incidence of NPC (23.1/100 000)[9], suggesting that improved diagnosis and registry may reveal additional "hot-spots" of high NPC incidence. Besides southern China, high incidence was also reported among Inuits and other native populations of the Arctic region.

Intermediate incidence rates of NPC are seen in Southeast Asia, including Singapore (15/100 000), Malaysia (9.7/100 000), Vietnam (7.5/100 000), Taiwan (7/100 000), and the Philippines (6.4/100 000). This trend also applies in Africa, including the eastern country Kenya (5.4/100 000) and northern countries Algeria, Morocco, and Tunisia (5.1/100 000)[10]. Therefore, epidemiologically, NPC is an interesting cancer because of this defined geographic and racial distribution, pointing to genetic, social, and environmental factors in the etiology of this tumor type. The incidence of NPC in other countries is generally low, and it is therefore considered a rare cancer in populations of the Americas, Japan, Korea, and Europe[1,11-14].

NPC is a frequent cancer in Indonesia, rating as the fourth most common tumor after cervical cancer, breast cancer, and skin cancer, and is the most common malignancy in the head and neck. The disease is 100% related to Epstein-Barr virus (EBV) infection, especially the most common undifferentiated type of NPC (WHO type III)[14,15]. In Indonesia, which has an ethnically diverse population of 225 million people, NPC is prevalent among different native people (s) and presents a major socioeconomic problem, with an overall incidence estimated at 6.2/100 000 or about 12 000 new cases per year[16]. Unfortunately, many of these cases go unregistered due to limited medical awareness as well as the lack of hospital facilities and a nationwide cancer diagnostic and registration system. Virtually all cases of NPC show genetic positivity for EBV, with multiple viral genes being expressed in each tumor cell[8].

EBV was identified by Sir Epstein and colleagues in 1964 in cells cultured from African Burkitt lymphoma tumor explants and the virus was subsequently found to infect more than 90% of the world population though was rarely considered pathogenic[17]. Primary infection usually occurs at childhood and is asymptomatic or presents as a mild inflammation or upper

respiratory infection[18]. In Indonesia, 100% of children at 5 years of age are infected with EBV and carry latent virus for life[19]. Delayed primary infection may cause a mild self-limiting illness known as infectious mononucleosis in adolescents and adults[4]. Occasionally, EBV infection may lead to chronic severe and fatal diseases, such as hemophagocytic syndrome and X-linked lymphoproliferative syndrome (Duncan's Syndrome). In humans, EBV infection initiates at the oropharyngeal epithelium upon transmission in the saliva. During this infection, the EBV virus infiltrates and transforms submucosal B lymphocytes that are important for viral latent persistence and further dissemination of the infection to distal epithelial surfaces including the nasopharynx[4]. After primary infection, EBV persists for life as a predominantly latent infection of B lymphocytes, with variable but persistent shedding in the saliva.

EBV is the first human virus to be linked to oncogenesis due to its close association with Burkitt lymphoma and subsequent linkage to the etiology of other lymphoid and epithelial malignancies. In immunosuppressed hosts, such as transplant recipients and untreated human immunodeficiency virus (HIV)-infected individuals, EBV is a major risk factor for the development of lymphoproliferative diseases, which may turn into malignant lymphomas if left untreated[20,21]. In immunocompetent individuals, the virus is associated with classic Hodgkin lymphoma, Burkitt lymphoma, and extranodal B and T/natural killer (NK) cell non-Hodgkin's lymphomas, as well as a few gastric adenocarcinomas and most undifferentiated and poorly differentiated NPCs worldwide. Patients (in developing countries) exposed to chronic inflammation and infection have been reported to be more prone to develop EBV-driven malignancies[21], a situation that might apply to Indonesia as well, but has not been analyzed in any detail yet. EBV is active in the malignant cells of all described tumor types, and each type of tumor has a distinct pattern of EBV gene expression. Individual EBV genes associated with the three main latency programs of EBV can contribute to the malignant phenotype, albeit in a different fashion in distinct tumor types[20,21]. EBV has been classified since 1997 as group 1 human carcinogen by the International Agency on Research on Cancer. Recently, the presence of latent EBV in tonsil epithelial cells was demonstrated[22], providing a basis for understanding the link between EBV and carcinogenesis. The data from that study support the model of dual epithelial-lymphoid tropism for the virus *in vivo*, indicating the possibility that healthy tonsil epithelium may play a role in transmission of the virus as part of the viral life cycle, and suggesting that EBV can play an initiating role in associated epithelial lesions like NPC and oral hairy leukoplakia. Transformation of epithelial cells into a malignant disease by EBV may be enhanced by environmental co-carcinogens, and premalignant dysplasia may progress rapidly into cancer[8,13,14,21].

NPC oncogenesis is not simply a consequence of EBV infection alone. More than 95% of adults in all ethnic groups across the world are healthy carriers of EBV. The transformation

of EBV infection into a malignant disease is probably a result of viral reactivation in combination with other (epi)genetic events, including the development of (multiple) cellular genetic lesions due to environmental carcinogens, food components, possibly combined with genetic immunodeficiencies[8,13,21]. Consistent with this hypothesis is the fact that NPC generally occurs several decades after primary EBV infection and NPC risk persists in first-line offspring[12,18]. On the other hand, many NPC cases are found in children and these cases generally have a more aggressive behavior, also suggesting a more direct role of EBV itself[23,24]. The geographic distribution clearly suggests a role for genetic and/or environmental co-factors. Therefore, the etiology of this disease appears to be multifactorial[10,13]. EBV infection, environmental factors (especially food), gender, and genetic susceptibility are consistent etiologic factors responsible for the higher incidence of NPC in certain ethnic groups, whereas other factors from air and soil, which depend more on the living environment of these groups, are less consistent. The parallel development of abnormal IgG and IgA antibody responses to EBV lytic antigens, which are characteristically associated with NPC development, most likely is a reflection of this process[18].

Because NPC in Indonesia has not been documented in much detail, we here present our clinical and epidemiologic observations on 1121 Indonesian NPC patients examined between 1995 and 2005.

CASE DEFINITION AND HISTORICAL ANALYSIS

All head and neck (H&N) cancer cases analyzed in this study were obtained from the archives of the Dr. Cipto Mangunkusumo General Hospital, which is a referral and teaching hospital located in the center of Jakarta that treats approximately 600 H&N cancer patients yearly[25]. All cases were histologically classified by standardized biopsy and staged according to the 2002 Union for International Cancer Control (UICC) criteria using clinical assessment and CT scan work-up. From an archive of more than 6000 H&N cancer cases registered between 1995 and 2005, we analyzed 1121 pathologically defined NPC cases treated at our hospital from which sufficient data were available. These cases include Indonesian citizens who are not all permanent residents of Jakarta, but also include patients who come from regional hospitals. As a referral hospital, we treat patients not only from Jakarta and surrounding areas but also from other islands and regions like Sumatra, Kalimantan, and Sulawesi. Almost 90% of the 1121 patients had been primarily diagnosed in our hospital; about 10% were referred from other hospitals in Jakarta and surroundings but were confirmed prior to intake in our hospital. Some patients from more distant underdeveloped rural areas lacked long-term follow-up. For overall pathologic data, we were able to access the combined pathologic database of 13 university hospitals in Indonesia compiled under the supervision of Professors Kurniawan and Cornain at our institute[25].

To obtain adequate data for investigation and follow-up, we selected 213 patients from these 1121 NPC patients as a separate study group. In these patients, more detailed analysis was done, including *in situ* hybridization for EBV-encoded RNA (EBER-RISH) using commercial kits (Dako or Novocastra) to prove EBV involvement. The results from this analysis were consistent with pathologic NPC classification. These patients lived in Jakarta and surrounding areas only, therefore enabling us to evaluate this subgroup more regularly and adequately with laboratory tests, which included determining the DNA viral load in nasopharyngeal brushings as well as whole blood samples and tests for EBV serology for IgA to virus capsid antigen-P18 (VCA-P18) and EBV nuclear antigen 1 (EBNA1)[26-28]. These tests were routinely performed at diagnosis, during treatment, and during follow-up after histological verification. Details of the EBV-related diagnostic results in our patients will be published elsewhere. In a selected group of juvenile and adult cases that were matched for TNM stage and sex and confirmed to be EBV positive by EBER-RISH using commercial reagents, we also analyzed the expression of latent membrane protein 1 (LMP1) using OT21C monoclonal antibody-based immunohistochemistry on paraffin-embedded tissue sections , as described before[29,30].

RESULTS

NPC incidence

From the intake registry in the Ear, Nose, and Throat department at Dr. Cipto Mungunkusumo Hospital, which includes 6000 H&N cancer cases registered between 1995 and 2005, we studied the incidence of individual cancer types, including 1121 cases diagnosed as NPC. The gender distribution among NPC cases showed 789 males versus 332 females. Because of incomplete patient records for the overall H&N cancer cases in the first five years, we could only evaluate the exact prevalence of NPC versus other H&N cancers from the year 2000 onwards (Figure 1). Of all H&N cancer patients treated between 2000 and 2005, including patients from referral centers in rural areas, the prevalence of NPC was around 28.35% (948 of 3344), followed by a 14.35% prevalence for skin cancer and 12.3% for lymphoid malignancies. The yearly incidence varied among tumors but the overall data consistently identified NPC as the most common H&N cancer in our institute for the 10-year period studied. Consultation with 13 other university hospital-based Ear, Nose, and Throat departments and the related pathologic databases throughout Indonesia confirmed this to be a consistent trend in the entire country (data not shown)[25].

In our cases, we found a similar predominance, with 70.4% male and 29.6% female cases yielding a 2.4:1 ratio. The male:female ratio was relatively stable over the years as shown in Figure 2.

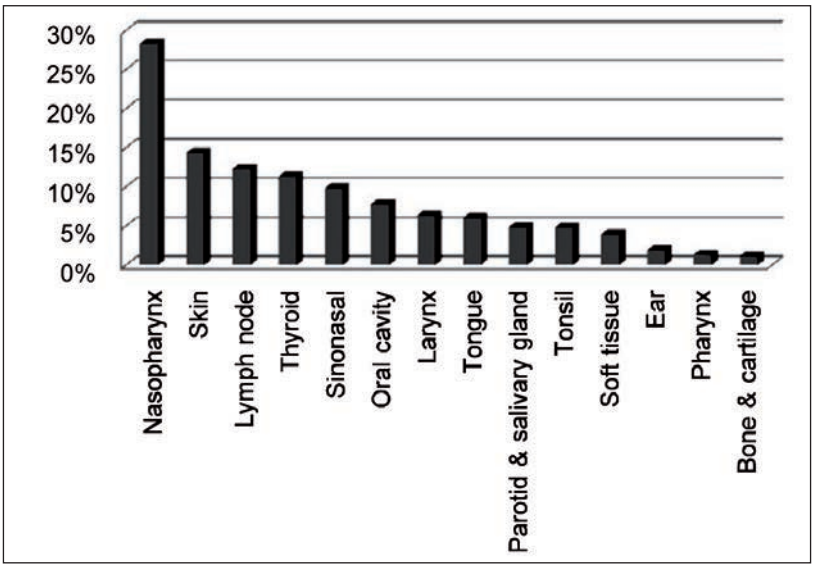

Figure 1 | Prevalence of nasopharyngeal cancer (NPC) and other defined malignancies among all head & neck cancer cases (*n* =3344) examined between 2000 and 2005 in the Dr. Cipto Mangunkusumo Hospital In Jakarta, Indonesia. NPC is the most prevalent head and neck cancer overall, representing about 28% of all cases.

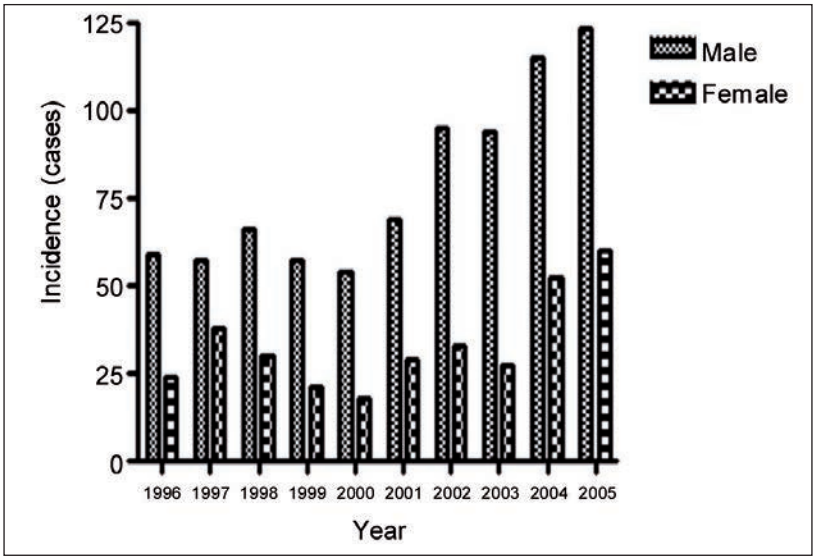

Figure 2 | Yearly NPC incidence (total number of NPC cases in the registry per year) and male and female predominance in the 1995–2005 period. The male-female ratio is rather stable over the years with an average of 2.6-fold male predominance. The increase in NPC incidence in recent years (2002 onwards) may be due to improved case definition and increased awareness.

Age distribution

NPC patients from various countries are described with ages ranging from 4 to 91 years, with a peak incidence at 50 to 60 years of age in Chinese populations. Generally, NPC is uncommon in individuals under the age of 20 years (less than 1%), whereas a bimodal age distribution has been described in northern Africa, with 20% of patients being below age 30[31-38]. As shown in Figure 3 and Table 1, the age distribution of NPC patients from our hospital had a peak at 40 to 49 years, and more than 80% of patients were diagnosed between 30 and 59 years of age. We observed a significant number (20%) of juvenile NPC cases, aged under 30 years, without a clear bimodal age distribution. Rather, our data showed a steady increase with age peaking at the fifth decade. For the younger age group, we found an intermediate yet stable incidence of 5 to 12 cases yearly (Figure 3 and Table 1). The higher incidence of juvenile NPC may reflect genetic susceptibility and/or young age exposure to co-carcinogens in the environment (to be detailed later).

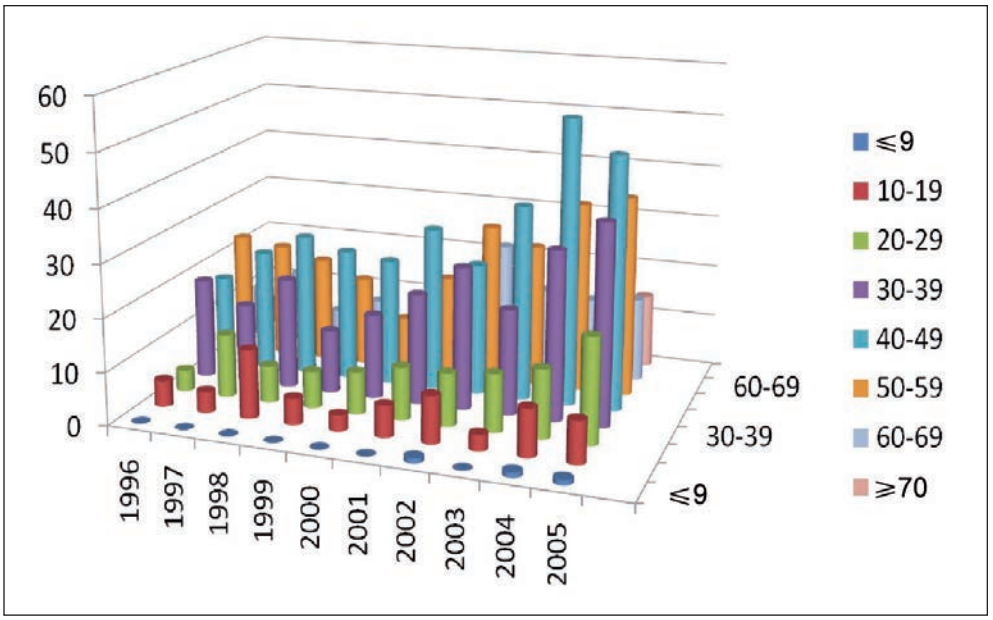

Figure 3 | Age distribution of the 1121 NPC patients in the 1995–2005 period. Although the NPC incidence in children of <10 years old is low, a considerable number of NPC cases were observed in the age groups 10–19 (Juvenile) and 20–29 (young adult) years old. Overall, the 1–30 year age group represented 21% of all cases. The NPC peak incidence in our cohort lays in the age groups of 30–50 years old, which is earlier than reported for Chinese populations. This illustrates that NPC in Indonesia affects the adult working population who carry responsibility for family and business, thus posing a significant socio-economic burden to the Indonesian society.

Table 1 | Age distribution in 213 consecutive local cases of nasopharyngeal carcinoma (NPC) in Dr. Cipto Mangunkusumo Hospital

Age (years)	Frequency	Percentage (%)
<20	21	9.9
21–30	25	11.7
31–40	50	23.5
41–50	69	32.4
51–60	35	16.4
61–70	9	4.2
>70	4	1.9

Table 2 | Estimated regional incidence of NPC per 100 000 registered inhabitants derived from 11 to 13 pathology centers in the year 2000

Center (region)	Incidence (/100 000)
Medan	4.30
Padang	2.22
Palembang	3.11
Bandung	7.00
Yogyakarta	4.90
Surakarta	4.93
Surabaya	7.23
Malang	9.19
Denpasar	8.41
Manado	6.54
Jakarta	4.41
INDONESIA	5.66

Two centers were excluded because of too little supportive data.

Regional NPC incidence

Due to the available Pathology Cancer Registry, which includes data from 13 different university hospitals in Indonesia, we were able to investigate the regional incidence of NPC in other areas in Indonesia as well. This registration system is the only access we have to cancer data in our country. Every university center or hospital has its own pathology cancer registration system, part of which is connected to this registry. Based on the data from this registry, we were able to study NPC incidence of 11 pathology centers in Indonesia that could provide sufficient data (Table 2). Because we investigated our subgroup from 1995 till 2005, we chose the year 2000 to evaluate a mean regional incidence. Bandung, Malang, Denpasar, Manado, and Surabaya represented high incidence areas, and therefore, early detection of

disease in these cities deserves special attention. Overall, the incidence of 5.66/100 000, equaling roughly 1000 new cases per month, reflected a major health problem in Indonesia, particularly because most of these patients were referred to the hospital at a late stage.

Ethnic origin

Table 3 shows the ethnic origin of our subgroup of 213 patients. Most patients were of Javanese origin, followed by Sundanese and Sumatranese people (30.5%, 25.8%, and 23.9%, respectively), confirming that not only Javanese but also other Indonesian ethnic groups were affected by NPC. More recent data for the 2007–2011 period from an ongoing treatment study at the radiotherapy department in our hospital confirmed that Javanese were the most prevalent ethnic group being treated for NPC (375/1173 patients, 32%), followed by Sundanese (19.2%), Chinese (10.6%), Batak (9.5%), Betawi (7.6%), Lampung (2.9%), and Minangkabau (2.4%) groups (Soehartati G, *et al*., personal communication). Although a strong genetic control is suggested for the disease, the incidence pattern among different ethnic groups in the Indonesian population showed no marked differences. For example, Chinese migrants have been shown to retain a high incidence of NPC even in subsequent generations, suggesting a strong genetic control. Our data reveal that in Indonesia, the disease does not follow Chinese demographics and does not seem to be influenced by Chinese genetics despite the large population of Chinese descendants living in Jakarta and its surroundings, and despite that a high NPC incidence with Chinese origin is indicated in neighboring countries, including Malaysia and Singapore.

Table 3 | Distribution of ethnic origin of 213 NPC patients from the Jakarta region

Ethnicity	Frequency	Percentage (%)
Java	65	30.5
Sunda	55	25.8
Sumatera	51	23.9
Betawi	25	9.9
Others	20	8.0
Chinese	4	1.9

Histopathology

According to WHO classification, NPC is histopathologically divided into three categories: keratinizing squamous cell carcinoma (WHO type I), non-keratinizing squamous cell carcinoma (WHO type II), and undifferentiated carcinoma (WHO type III). NPC WHO type III, is the most prevalent form of NPC in Southeast Asia and other high incidence regions, and is

most closely associated with EBV infection. WHO type I tumors can also be associated with EBV in endemic regions, but usually not in non-endemic regions, where they result from tobacco and alcohol abuse and are EBV-negative[39,40].

WHO type III was the most frequent histopathologic type in our study population. Around 85.0% of cases proved to be undifferentiated carcinoma. Interestingly, our population contained 12.7% WHO type I NPC tumors (classification confirmed via blind reassessment by independent pathologists), all of which were EBV-positive by EBER-RISH. Only 2.3% of the cases were classified as WHO type II NPC tumors.

Table 4 | Expression of Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) in juvenile and adult NPC cases

Item	Cases	Frequency of LMP1 expression			*P*
		Mean ± SD	Median	Range	
Age					0.795
<30 years	24	2.9 ± 4.0	0.7	0 – 11.7	
≥30 years	24	1.8 ± 2.3	0.8	0 – 8.4	
Sex					0.950
Male	28	2.7 ± 3.8	0.6	0 – 11.7	
Female	20	1.9 ± 2.5	0.9	0 – 8.4	
WHO histopathologic type					0.364
WHO I	8	2.8 ± 2.9	2.5	0 – 8.4	
WHO III	40	2.3 ± 3.4	0.6	0 – 11.7	
WF histopathologic type					0.364
KS	8	2.8 ± 2.9	2.5	0 – 8.4	
Type A	40	2.3 ± 3.4	0.6	0 – 11.7	
Analysis stage					0.589
Early	8	1.0 ± 1.2	0.8	0 – 3.6	
Advanced	40	2.6 ± 3.5	0.7	0 – 11.7	
T stage					0.042
T1	6	1.8 ± 1.6	1.85	0 – 3.6	
T2	15	2.5 ± 3.6	0.8	0 – 10.5	
T3	9	1.4 ± 0.8	0.1	0 – 4.4	0.039 (T1–T3)
T4	18	3.6 ± 4.0	1.7	0 – 11.7	0.007 (T3–T4)
N stage					
N0	4	2.0 ± 3.8	0.1	0 – 7.6	
N1	12	1.8 ± 2.7	0.9	0 – 9.6	0.553
N2	12	1.7 ± 2.3	0.5	0 – 7.8	
N3	20	3.2 ± 4.1	0.8	0 – 11.7	
M stage					0.706
M0	42	2.4 ± 3.4	0.8	0 – 11.7	
M1	6	1.7 ± 3.1	0.6	0 - 8.0	

LMP1 expression level was assessed on paraffin tissue sections with OT21C mouse anti-LMP1 monoclonal antibody staining, using the scoring method as detailed elsewhere[30].

In our selected 213 cases, we also studied the differences of EBV-LMP1 expression in NPC between patients of < 30 years (*n* = 24) and ≥ 30 years old (*n* = 24), matched for sex and tumor TNM stage (Table 4). LMP1 expression was detected in 160 (75%) cases with a staining intensity score ranged from 0 to 11.7. The average score for patients of < 30 years old was higher, but was not different significantly ($P > 0.05$). There was a borderline significant relationship between LMP1 expression and T stage ($P = 0.042$), but not with N and M stages. The intensity score of EBV-LMP1 expression in this study was somewhat lower than others[30]. Higher LMP1 expression in patients < 30 years old was associated with more locoregional progressivity at young age.

Etiology

Early age EBV infection and chronic viral reactivation in nasopharyngeal epithelial tissues due to locoregional inflammation may be fundamental for NPC development. In this respect, it should be noted that nearly 100% of Indonesian children are EBV carrier at age 5[19]. Many environmental factors are considered important for NPC development. Dried salted fish, common in the Indonesian diet, have been reported to cause NPC due to the nitrosamine content[10,41,42]. Chronic exposure to and intake of chemical carcinogens, formalin and phorbol esters, that are also widely spread in Indonesia, are considered as important risk factors as well, although little detail is known yet[43,44]. A reflection of chemical co-carcinogenesis may presented by high levels of genome methylation, as recently described in Indonesian NPC patients and regional controls[45]. A number of studies have reported familial linkage for NPC risk, suggesting genetic susceptibility. However, in our 1121 NPC cases, we did not find any familial association. A number of reports have suggested a role for histocompatibility complex (HLA), in combination with EBV mutant strains, in NPC. HLA linkage data reveal that younger and older onset patients are genetically different and may involve different mechanisms[1012]. Recent genome-wide linkage analyses of high-risk Chinese familial NPC pedigrees identified two candidate NPC susceptibility loci, 4p15.1-q12 and 3p21.3, with another suspected locus reported at 5p13-15[8,46-54]. However, more recent large-scale studies with appropriate local non-NPC controls have cast doubt on these early findings, and no clear NPC-related EBV strain nor (limited number) genetic marker has been identified as outstanding entity[51].

Clinical signs and symptoms at presentation

Most patients in our study cohort presented with advanced disease. Early stage NPC is difficult to diagnose clinically because of its hidden localization in the nasopharynx. Misdiagnosis could also result from patients who lack of knowledge about early signs and symptoms of NPC and cancer in general. Denial of cancer diagnosis and economical restrictions may delay medical treatment. On the other hand, doctors also contribute to late

NPC diagnosis because of ignoring or misdiagnosing the unspecific symptoms mimicking upper respiratory tract infection during early stages. A recent study confirmed the poor awareness of NPC early signs and symptoms among regional health workers in Indonesia[55]. Basically, examination and biopsies of the tumor and the nasopharynx need to be performed by a direct nasoendoscopic examination (preferably using flexible fibreoptic endoscope). This is one of the most important skills required for the diagnosis and monitoring of NPC and may facilitate accurate brush sampling in the nasopharyngeal space to assess EBV-DNA load, which appears closely linked to local presence of NPC[27]. In high-risk regions, doctors should be more aware of early-stage, unspecific signs and symptoms to improve recognition, diagnosis, and downsizing of tumors at presentation, thereby improving treatment options. With this in mind, we ranked the most common signs and symptoms of Indonesian NPC patients in our study, by compiling the results from a questionnaire completed on patient intake (Figure 4).

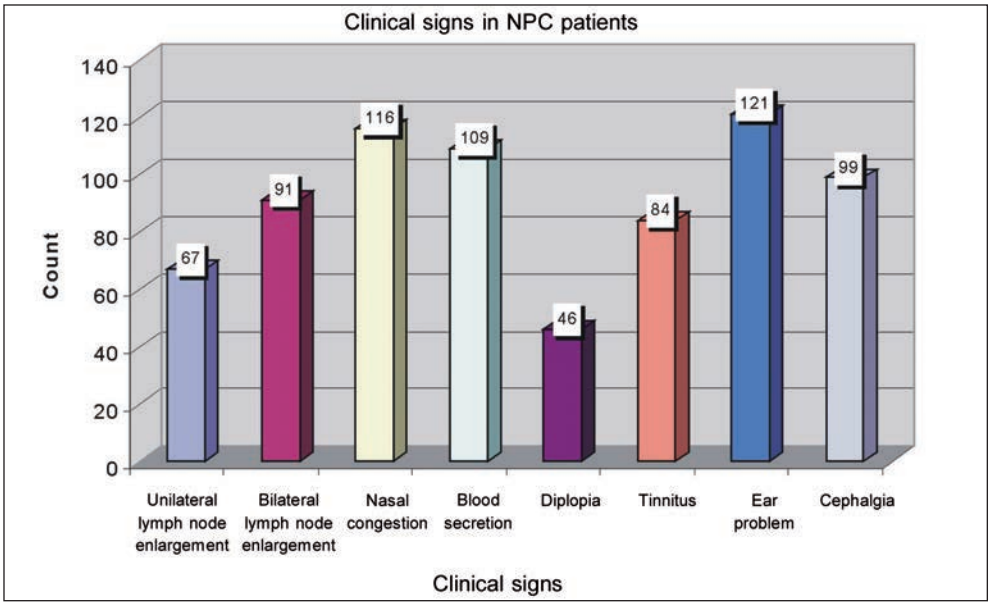

Figure 4 | Most predominant signs and symptoms at first presentation in 733 of the 1121 NPC patients. It may be deduced from this graph that most common symptoms associating with first presentation are generic for many other ear, nose, throat syndromes and cannot be taken as being characteristic for NPC. However, doctors confronted with patients having a combined or chronic history of these symptoms, without relief by conventional (anti-bacterial, anti-allergic) therapy, should be on alert for more detailed investigation at early stage, including nasendoscopy and EBV-IgA serology[28]. Upon persisting symptoms and abnormal positive EBV-IgA serology novel non-invasive diagnostic procedures, like nasopharyngeal brushing combined with EBV-DNA measurement[27], may be indicated and informative for early detection of NPC as underlying cause of symptoms.

Most of our patients (60.6%) had recognized they already had a unilateral ear problem, the earliest sign of NPC, several months before diagnosis. Second and third most prevalent symptoms at presentation were persistent nasal congestion and nasal blood secretion. However, our data indicated that neither patients nor doctors gave this condition attention until cervical lymph node enlargement, a sign of late stage NPC, was detected.

DISCUSSION

Here, we presented our data about the incidence of NPC in Indonesia using pathologic reports from 13 university centers in Indonesia collected in the Pathology Cancer Registry System. We also evaluated, in Dr. Cipto Mangunkusumo Hospital, NPC incidence in all patients treated between 1995 and 2005 and assessed the epidemiologic and clinical data of 213 patients in which additional markers for NPC were examined. Overall, our data on NPC prevalence for Indonesia are comparable to those reported in 1998 by Soeripto[16], indicating a prevalence of around 6 /100 000, which is in line with the Globocan-IARC estimates as reported[1,11].

From the above mentioned data, it is clear that Indonesia is still an unexplored region with considerable NPC incidence, yielding about 12 000 new NPC cases on a yearly basis. Denpasar, Malang, Surabaya, and Bandung are, for example, regions of high incidence. Acquired data for these regions are poor in detail, suggesting these regions should be explored more intensively. Although most patients in our study are of Javanese origin, it became clear that other ethnic groups in the overall population of Indonesia are also affected by NPC. Importantly, upperclass Indonesian patients may seek specialized treatment in neighboring countries like Singapore, Malaysia, and China. It is appreciated that these may include patients of Chinese origin, but this is unlikely to influence the overall results on ethnic origin. Therefore, NPC is a major multi-ethnic problem in Indonesia and not only linked to Chinese genetics. A prior study by Devi *et al*.[9] in the province of Sarawak, Malaysia showed that the age-adjusted incidence in Sarawak residents was 13.5/100 000 (95% CI: 12.2–15.0/100 000) in males and 6.2/100 000 (95% CI: 5.7–6.7/100 000) in females. The risk in the Bidayuh people was 2.3-fold (males) and 1.9-fold (females) higher than the Sarawak average and about 50% higher than those in other regional populations. The high risk in native people of Sarawak, however, is unlikely to result from blending with citizens of Chinese descent that form a distinct ethnic group in Malaysia, similar to Singapore. These findings and data from this study suggest NPC risk to be endogenous to the local population in Southeast Asian multi-ethnic countries, including Indonesia. The observed increase of NPC cases in our institute in recent years may be due to improved referral rather than true incidence. This

may be related to improved awareness and implementation of more advanced treatment options, in particular in the Jakarta region.

The age distribution of NPC in Indonesia is different compared to previous data from China and North Africa. A similar age distribution has also been reported by Loh *et al*.[36], who showed that of 323 new patients treated between 1998 and 2004 in the National University Hospital in Singapore, 36% to 40% were diagnosed at 41 to 50 years of age. In the literature, an overall peak incidence is described at 50 to 60 years of age. In high risk areas, such as Hong Kong, the NPC incidence in each sex rises sharply from the age of 20 onward and also reaches a plateau between 40 and 60 years of age[33]. In addition to this peak incidence at middle age, a second peak incidence is described in the literature for a younger age group, 10 to 29 years. This peak incidence is particularly found in northern African countries and some Chinese populations as well[37,38]. In China, the overwhelming majority of the cases occur in the fifth and sixth decades of life. In contrast, there is a bimodal distribution in North Africa, with a major peak incidence around 50 years of age, similar to the single peak observed in China, and a minor peak in people aged between 10 and 25 years old. This juvenile form accounts for approximately 20% of the patients and has specific clinical and biological features[3]. Jeannel *et al*.[10] reported that the age-specific incidence for NPC differs from other tumor types affecting older age groups. Whereas the peak incidence for other tumors is reached around the age of 45 to 49 years, the incidence of NPC is approximately stable until 60 to 64 years of age, after which it declines. In Indonesia, a steady increase is observed well before the age of 45, starting at early adolescence. Age distribution for NPC is bimodal in some northern American populations and in the Mediterranean region, with a peak incidence at 10 to 20 years and a second at 40 to 60 years of age. Children under 16 years of age account for 1% to 2% of all patients with NPC in China, 2.4% in the United Kindom, 7.12% in Turkey, 10% in the United States, 12% in Israel, 13% in Kenya, 14.5% in Tunisia, and 18% in Uganda[14]. In Indonesia, 17% to 21% of all patients are under the age of 30, as observed over a 10-year period. Our data on the overall NPC incidence did not differ significantly among regional centers in the larger Jakarta area and were also similar to those obtained in the Dr. Sardjito Hospital at the Gadjah Mada University in Yogyakarta, a more rural region of Mid-Java, where 450 cases were recently analyzed (Hariwiyanto B; unpublished data and personal communication).

Previous epidemiologic studies suggest three major etiologic factors for NPC: genetic susceptibility, early age exposure to chemical carcinogens (particularly Cantonese salted fish), and latent EBV infection[7,8,41,42]. Preserved foods other than salted fish could also play a part in the etiology of NPC and methods of cooking may have an effect on the amount of volatile nitrosamines ingested[41]. In Malaysian Chinese, the consumption of beef liver,

in addition to salted fish and salted eggs, appeared significantly associated with NPC. In addition to these factors, the presence of nitrosodiethyl amine in smoke and dried meat and the use of herbal nasal medicine are well-known risk factors of NPC. Also, improperly (formalin-treated) preserved foodstuffs, which are rather common in Indonesia[43,44], may be important as etiologic risk factors. Another risk factor is environmental inhalants, a significant number of which have been reported to be associated with NPC. These include fossil fuels from cooking due to smoke and fumes from wood, which contains significant quantities of benzopyrene, benzanthracene, and polycyclic aromatic hydrocarbons. Another source of carcinogenic hydrocarbons is textile dyes, which are still in common use in local Indonesian markets for food coloring. The consumption of some herbal teas, and in particular teas containing Euphorbia family plant extracts, is considered a risk factor. Occupational exposure of formaldehyde also increases the risk. Finally, smoking cigarettes with exotic additives and working in poorly ventilated places are strongly associated with NPC. Interestingly, the widely spread use of incense burning in Southeast Asia has not yet been considered as a risk factor.

Preserved vegetable intake is associated with a 2-fold increase in NPC risk, whereas high non-preserved vegetable intake is associated with a 36% decrease, consistent between vegetable types and countries. Direct measurements of N-nitroso compounds from preserved foods collected in regions of high and low incidence as well as in different areas within a high incidence region did not correlate with the regional and local variations in incidence. In contrast, preserved and fresh foods consumed in developing and Western countries contain very low levels of N-nitroso compounds[10,13]. The content of N-nitroso compounds in Indonesia has not been evaluated yet.

Epidemiologic studies point to the protective role of regular consumption of fresh fruits and vegetables, presumably because of the vitamin content, especially vitamin C. Vitamin C may act in blocking either nitroso compound metabolism or EBV reactivation. Activation of EBV *in vitro* by tumor promoter TPA (12–0 tetradecanoyl phorbol-13-acetate of the phorbol ester family), which has EBV lytic cycle-inducing capacities, can be inhibited by vitamin C[10,37]. Furthermore, besides the widespread consumption of dried salty fish, it is rather common in Indonesia to find known carcinogens like formalin and polyaromatic chemical dyes in the food supply at local markets and small factories[43,44]. Furthermore, cigarette smoking and “therapeutic” inhalation of various aromatics are rather common in Indonesia, adding to the co-carcinogen burden from the environment. Chronic exposure to these (co-)carcinogenic factors and EBV latent infection may increase, in synergy, the risk for NPC development. Chronic exposure to co-carcinogenic compounds may be reflected in increased methylation of defined tumor suppressor genes, as recently revealed by us and others[45,50].

EBER *in situ* hybridization (EBER-RISH) is considered the gold standard for detecting and localizing latent EBV in tissue specimens, whether frozen or formalin-fixed and parafin-embedded[56,57]. This test is the most reliable method for determining if a lesion is EBV-associated and is used diagnostically in several specific clinical situations. In biopsy, EBER-RISH is often helpful in differentiating infectious mononucleosis, Hodgkin's disease, and/or non-Hodgkin's lymphoma and to define EBV involvement in the pathogenic process. It is also used routinely for confirming a diagnosis of EBV-driven postransplant lymphoproliferative disorder (PTLD)[58]. Further analysis using EBER-RISH is warranted to define the overall impact of EBV involvement in Indonesian H&N cancers including NPC. However, EBER-RISH is an expensive and complex procedure, not well suited for routine application under sub-optimal laboratory conditions[56]. Likewise, a biopsy from the nasopharyngeal space is a painful and invasive procedure and tissue processing may not be generally available. Therefore, current efforts are on defining non-invasive diagnostic procedures based on EBV-DNA detection in blood, plasma, or nasopharyngeal brushings[26-28,56]. Additionally EBV-IgA serology may prove suitable for early identification of individuals at risk (family members) or at early stages of NPC[36].

These novel approaches are becoming increasingly available and may ready for large scale (screening) in the near future, which will be of particular relevance to developing countries with medium-high NPC incidence like Indonesia.

NPC is ranked fourth among cancers in males in Indonesia. Patients are generally referred at a late stage. The overall treatment is complex, not cost-effective, and places a significant socio-economic burden onto patients and their families. Adequate data for follow-up from referral centers are usually not available. Registration of patients with NPC is, in most cases, not digital and therefore inadequate. Thus, it is difficult to compare treatment results from several centers and even more difficult to compare treatment results with other countries or to include patients in protocols for international studies. Patients are often referred to the hospital at a late stage, which has a major drawback on their prognosis. As a result, even many young patients are treated for late-stage disease and unfortunately become victims of a deadly disease at a young age. There is no doubt that this disease, affecting individuals at 40 to 50 years of age as well as those under 30 years old, represents a large socio-economic burden for the country and its health system. Therefore, early detection by simple and affordable techniques, such as nasopharyngeal brushing[27] and blood investigations[26,28] and adjuvant laboratory examinations, for regular assessment of the status of disease-specific markers, are of utmost importance. Molecular testing, such as peptide-based EBV-IgA serology and EBV-DNA load testing, holds promise for early detection and down-staging NPC in Indonesia when applied on a country-wide scale. The availability of simple sampling and

stabilized transport options is relevant for collection of clinical specimens at remote (rural) health centers[59]. Finally, the need for and importance of adequate digital early registration of patients for treatment and follow-up of NPC also cannot be overestimated.

NPC remains one of the most confusing and commonly misdiagnosed diseases. There are multiple non-specific early signs and symptoms of NPC, but they can be taken as early warnings for doctors to improve awareness and send samples for specific testing. Educating regional health workers and hospital staff is a critical first step for controlling NPC at early stage.

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Chapter 3

Non Invasive diagnosis of nasopharyngeal carcinoma: nasopharyngeal brushings reveal high Epstein-Barr virus DNA load and carcinoma-specific viral BARF1 mRNA

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is the most prevalent ENT-tumour in Indonesia. We investigated the primary diagnostic value of EBV DNA load and mRNA detection in non-invasive nasopharyngeal (NP) brushings, obtained prospectively from consecutive Indonesian ENT-patients with suspected NPC (N=106) and controls. A subsequent routine NP biopsy was taken for pathological examination and EBER-RISH, yielding 85 confirmed NPC and 21 non-NPC tumour patients. EBV DNA and human DNA load were quantified by real-time PCR. NP brushings from NPC patients contained extremely high EBV DNA loads compared to the 88 non-NPC controls ($p<0.0001$). Using mean EBV DNA load in controls plus 3 SD as cut-off value, specificity, sensitivity, positive and negative predictive values were 98%, 90%, 97% and 91% respectively. EBNA1 and the carcinoma-specific BARF1 mRNA were detected by NASBA and found in 86% and 74% of NP brushings, confirming NPC tumour cell presence. EBV RNA positivity was even higher in fresh samples stored at-80ºC until RNA analyses (88% for both EBNA1 and BARF1). EBV RNA-negative NP brushings from proven NPC cases had the lowest EBV DNA loads, indicating erroneous sampling. No EBV mRNA was detected in NP brushings from healthy donors and non-NPC patients. In conclusion, EBV DNA load measurement combined with detection of BARF1 mRNA in simple NP brushings allows non-invasive NPC diagnosis. It reflects carcinoma-specific EBV involvement at the anatomical site of tumour development and reduces the need for invasive biopsies. This procedure may be useful for confirmatory diagnosis in large serological NPC screening programs and has potential as prognostic tool.

INTRODUCTION

Undifferentiated nasopharyngeal carcinoma (NPC WHO type III) is virtually 100% associated with Epstein-Barr virus (EBV) and has a reported high incidence in most of South-East Asia and intermediate incidence in North-African populations and in Inuit[1-3]. In Indonesia, with an ethnically diverse population of 225 million people, NPC is the most common ENT tumour with high prevalence among native populations and a yearly overall incidence estimated at 6.2/100,000[4]. Extremely high incidence was recently documented in native populations living on the island of Sulawesi[5]. In Yogyakarta, Central-Java, NPC is the most prevalent tumour among man and 4th most prevalent among females, with a male/female ratio of 2.4, constituting respectively 22% and 8% of all diagnosed malignancies[4].

The strong etiological link between EBV and NPC has been known for over 3 decades[1-3,6] and is reflected by abnormal anti-EBV antibody profiles, increased circulating EBV DNA levels and by distinct EBV gene expression in the tumour cells[7-11]. Classically, NPC is considered to have a latency type 2 EBV transcription, with expression of EBV-encoded small RNAs 1 and 2 (EBER1/2), BamHI A rightward transcripts (BARTs), Epstein-Barr nuclear antigen 1 (EBNA1) and latent membrane protein 2 (LMP2), while LMP1 is more heterogeneously expressed[2,12,13]. Previously, we and others showed additional transcription of a viral oncogene encoded in the BamHI-A rightward frame 1 (BARF1)[10,14,15]. BARF1 mRNA is exclusively expressed in EBV-positive carcinomas (i.e. NPC and EBV-positive gastric carcinomas) and is virtually absent from EBV-linked lymphoma[10,15]. BARF1 has transforming activity *in vitro* and *in vivo*[16-18]. BARF1 encodes a 33KDa type-II membrane protein that can be cleaved after amino acid 20 to release a soluble 29 kDa fragment with mitogenic activity, whereas the remaining transmembrane domain increases bcl-2 expression, thus contributing to cell growth and survival. Recent reports further substantiated that BARF1 protein is rapidly and efficiently secreted by epithelial cells[19-21]. Currently, diagnosis of WHO type III NPC requires a biopsy from the primary tumour site or metastases for histopathological assessment and demonstration of Epstein-Barr virus (EBV) involvement by *in situ* hybridisation for EBER1/2. Even at the early stages of NPC, patients are characterised by aberrant serological responses to EBV compared to healthy EBV-carriers. Both IgG and IgA antibody responses to defined EBV proteins may be used as NPC markers[11]. Quantitation of circulating EBV DNA may be useful for prognostic monitoring in a subset of patients[9], but because of low or negative EBV DNA values in a significant number of NPC patients, this method is less suited for primary diagnosis[22]. EBV-DNA in blood of NPC patients appears highly fragmented reflecting tumour apoptosis or necrosis[22,23].

Because serology and quantitation of circulating viral DNA only indirectly reflect carcinoma-associated activity of EBV in the nasopharyngeal region, we further explored the consistent etiological link between NPC and EBV in this study. We hypothesized that NPC may be more directly reflected by elevated viral DNA levels plus carcinoma-specific viral transcriptional activity at the site of the primary tumour development. This is indicated by recent reports, showing elevated EBV DNA loads in nasopharyngeal (NP) swab samples of NPC patients using non-standardized PCR techniques[24-27]. Thus, we investigated in more detail the diagnostic value of EBV DNA load quantification and EBV mRNA detection, in particular BARF1 and EBNA1 mRNA, in NP brushing specimens from consecutive Indonesian ENT patients with suspected NPC and various controls. In all patients suspected for NPC a biopsy was taken from the same site to confirm presence or absence of NPC by using EBER in situ hybridisation (EBER-RISH) and immunohistochemical staining for EBNA1 and LMP1. We used a clinically well-validated LightCycler-based real-time PCR for rapid and accurate EBV DNA load determination[28,29] and NASBA assays for sensitive and specific detection of viral mRNA in high EBV DNA backgrounds[10,15]. RT-PCR was used in addition to confirm the presence of spliced EBV mRNA. This combined EBV DNA/mRNA approach may enable diagnosis of NPC by directly revealing aberrant tumour related EBV activity at the anatomical site of NPC tumour development. This non-invasive procedure may allow objective NPC diagnosis and may reduce the number of invasive biopsies required. It may also be used as confirmatory test in serological screening programs and is a putative prognostic tool.

MATERIALS AND METHODS

Patients

NPC patients (N=85) were identified at Sardjito Academic Hospital, Gadjah Mada University School of Medicine, (Yogyakarta, Indonesia) in a population of 106 ENT-patients suspected of having NPC based on first clinical examination in the period 2002-2004. Diagnosis was based on pathological assessment of paraffin-embedded tumour biopsy specimens, EBER1/2 *in situ* hybridisation using commercial PNA-based hybridisation probes (Dakocytomation, Glostrup, Denmark) and immunohistochemical staining for EBNA1 and LMP1, using previously described monoclonal antibodies[30,31]. Approval of the local medical ethical committee was obtained for this study and all patients and controls signed for informed consent. TNM staging was done for all patients as described previously[32] using clinical measurements and CT scans as part of the routine work-up.

Cell lines

The EBV-positive NPC cell line C666-1 (kindly provided by Dr. Dolly Huang), the EBV-positive marmoset lymphoblastoid cell line (LCL) B95-8 and the human LCL JY were used as positive control for RNA amplification. Besides EBV latent RNAs, both LCLs express B-cell associated lytic cycle EBV RNAs in a minority of cells, including BARF1 mRNA. The C666-1 NPC cell line has a latent EBV transcription phenotype including BARF1 mRNA expression. The EBV-negative Burkitt's lymphoma cell line Ramos (ATCC CRL-1596) was used as negative control.

NP brushing samples

NP brushing was performed by experienced ENT-specialists and ENT resident trainees. In all cases the nasopharyngeal (NP) brush sample was taken prior to the biopsy in patients with suspected NPC, and both were sampled from the same site, as defined by nasendoscopy. In total, 85 NP brush samples were obtained from patients with subsequently biopsy-proven EBER-positive NPC. Suspected NPC patients who yielded an EBER-negative biopsy but were diagnosed with ENT malignancy were included as non-NPC tumour controls. In total, 88 control NP brushings were sampled, obtained from 21 patients with non-EBV-associated head and neck carcinomas and 22 patients with other otorhinolaryngological complaints; as well as from healthy EBV-seropositive donors from the Yogyakarta region (N=28) and Amsterdam (N=15) and 2 EBV-seronegative donors.

Nasendoscopy-guided NP brushings were taken after applying local anaesthetic spray (1% Lidocaine; AstraZeneca, Waltham, USA). A flexible nasendoscope was used to evaluate the entire nasopharynx and the site of tumour involvement. For the brushing, a standard Cytobrush Plus (Medscand, Malmö, Sweden) with a wire shaft was used, which was contained in a plastic catheter covering the entire shaft of the brush, to prevent contamination of cells from non-nasopharyngeal sites. The catheter with brush was inserted via the nose until the nasopharynx was reached. Then the brush was released from the catheter and the cytobrush was gently rotated for several times over the nasopharyngeal epithelium, returned into the catheter and removed. Subsequently, for an initial series of samples, 2 smears were made on glass slides for cytological evaluation and then the cytobrush tip (~1.5 cm) was cut-off and placed in 4 ml of NucliSens lysis buffer (BioMerieux, Boxtel, The Netherlands), mixed well and stored at -80ºC until use. This buffer instantly stabilizes DNA and RNA, permitting short-term transport and storage at ambient temperatures and enabling long-term preservation at -80°C[33,34].

EBV parameters in the brushing specimens were analysed batch wise in a blinded fashion at the department of Pathology, VU Medical Centre, Amsterdam, the Netherlands, uninformed

about the NPC status of the samples. For this purpose NP brushing samples collected in Indonesia and stored in lysis buffer within 2 hours after collection were sent on dry ice to Amsterdam in 2 batches. The first arrived in thawed condition, whereas the second was kept on dry ice during the entire transport. Furthermore, in order to yield as many NPC cells as possible for DNA and RNA analyses, brushes from the second batch were directly put in NucliSens lysis buffer without preparing glass slides for cytology. This was also done for the post-therapy follow-up samples.

Blood and serum samples

Blood samples (9 ml) were drawn at the time of NP brushing/biopsy collection and 0.5 ml was mixed immediately with 4.5 ml NucliSens lysis buffer and stored at -80C. The remaining blood was used for preparation of serum by clotting at +4C overnight and subsequent removal of the clot by centrifugation. Serum was aliquoted and stored at -20C.

Nucleic acid isolation by silica-based extraction

DNA and RNA were simultaneously isolated from NP brushing samples by silica-based nucleic acid extraction as described previously[35]. One ml of lysate was used as input for the isolation procedure and the nucleic acids were eluted in 100 µl of elution buffer. Reagents for the isolation procedure were obtained from BioMerieux, Boxtel, the Netherlands.

EBV DNA load and cellular DNA quantification by quantitative LightCycler-based (LC) real-time PCR assays

EBV DNA load in NP brushings was determined by a quantitative LC-based real-time PCR targeting a highly conserved 213 bp region of EBNA1, a single copy gene of EBV. This assay was described in detail elsewhere[28,29].

The amount of human diploid genome equivalents in NP brushing specimens was determined by quantitative LC-PCR targeting a 197 bp fragment of the human β-globin gene[36]. Five µl of nucleic acid eluate was used as input for all PCR assays. β-globin DNA-negative samples (N=1) were excluded from the study.

Nucleic acid sequence based amplification (NASBA) for EBV RNA detection

NASBA is a sensitive, isothermal RNA amplification technique which enables specific RNA amplification in a high DNA background, regardless of RNA splice patterns[37]. NASBA reagents were obtained from BioMerieux (NucliSens basic kit, BioMerieux, Boxtel, the Netherlands).

Oligonucleotide primers for LMP2 and (non-spliced) BARF1 mRNA were described before[10,15]. EBNA1 NASBA primers were located within the (non-spliced) open reading frame of this gene, enabling simultaneous amplification of all putative splice variants and EBNA1 transcripts derived from the C, W, Q or F promotor[10,15]. Analytical and relative sensitivities of the NASBA assays were previously determined at 10 mRNA molecules and an RNA amount equivalent to <1 EBV-infected cell respectively[10]. Finally, for detection of A3/A4-spliced BART RNA we used an RT-PCR assay[38]. Several precautions were taken during PCR and NASBA to avoid false positivity, as described previously[29]. In all experiments appropriate negative and positive controls were included during nucleic acid isolation and amplification.

EBV serology

All ENT patients and healthy controls were analysed for serum IgG and IgA antibodies to EBV-specific and immunodominant VCA-P18 (BFRF3) and EBNA1 (BKRF1) epitopes using synthetic peptide-based ELISA tests and by immunoblot for EBV-specific IgG and IgA antibody-diversity profiling, exactly as described before[11].

Healthy controls were characterised by restricted IgG antibody diversity profiles with IgG antibodies to VCA-p18 and/or EBNA1, but rarely had EBV-specific IgA antibodies. NPC patients were characterised by positive IgG and IgA responses to VCA-p18 and EBNA1 epitopes and all had an abnormal IgG diversity profile in immunoblot, used as confirmatory test[11].

RESULTS

NP brushings of NPC patients contain extremely high EBV DNA loads

The NP brushing procedure was well tolerated and none of the patients or controls complained of any negative effects caused by the brushing procedure, except for nasal dripping or relative mild bleeding in approx. 40% of suspected NPC patients and an occasional healthy control, which was nowhere comparable to the excessive bleeding and pain often observed during and after taking the nasopharyngeal biopsy. The use of a flexible endoscope facilitated proper sampling at the site of suspected tumour mass.

To assess whether the brushing procedure was a reproducible approach to collect cells from the nasopharynx, the amount of human diploid genome equivalents was determined first by LightCycler PCR in sixty-one NP brushing samples (48 NPC patients and 13 controls). This yielded a highly consistent and comparable value for NP brushings of both NPC patients and controls (mean $5.3*10^6$; range $4.8*10^5$ - $2.9*10^7$), indicating that the NP brush procedure

is a reliable and reproducible means of sampling (figure 1). No difference was observed for the amount of human diploid genome equivalents in NP brushings from NPC patients versus controls (Mann-Whitney test, p=0.21).

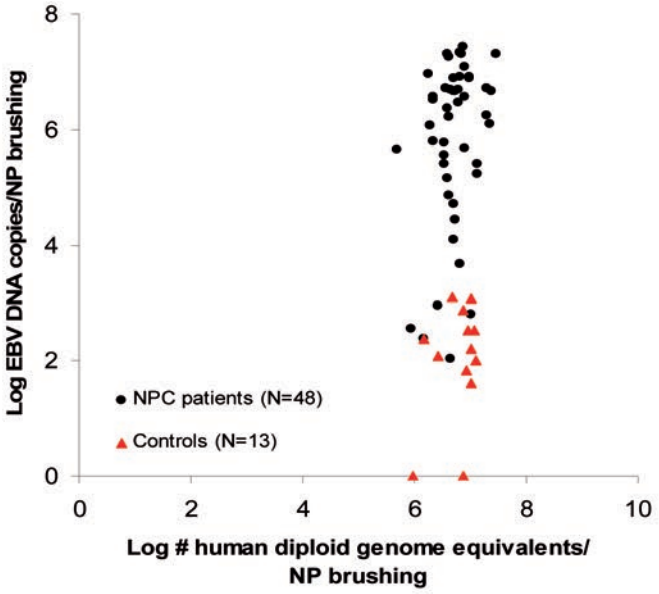

Figure 1 | NPC patients and controls have similar numbers of human diploid genome equivalents/NP brushing, as determined by LightCycler-based real-time PCR for the human β-globin gene. EBV DNA load in nasopharyngeal brushing samples ("EBV DNA copies/NP brushing") is unrelated to the number of diploid genome equivalents.

Quantitative analysis of EBV DNA copies was performed using a standardised and well-validated LightCycler-based real-time PCR for the single copy EBNA1 gene. All NP brushing samples (100%) from NPC patients (N=85) were positive for EBV DNA, with extremely high viral DNA loads (median: $2.37X10^6$ EBV DNA copies/brushing; mean: $5.45X10^6$ EBV DNA copies/brushing; range $1.08X10^2$ - $4.88X10^7$; figure 2).

In the NP brushings from the control population (N=88 in total), EBV DNA was detectable in 68/88 cases (77%) but the EBV DNA load was very low ranging from 0-4,158 EBV DNA copies/NP brushing only (median: 177; mean: 376 EBV DNA copies/NP brushing). A statistically significant higher EBV DNA load was observed in NPC patients compared to controls (Mann-Whitney test $p<0.0001$), with only marginal overlap between these 2 groups, as shown in figure 2. In the controls, consisting of 21 non-NPC tumour patients, 22 patients with other ENT-complaints and 45 healthy donors, no differences were observed in the mean values

of EBV DNA loads between the different populations, which were similarly low in all groups (see figure 2).

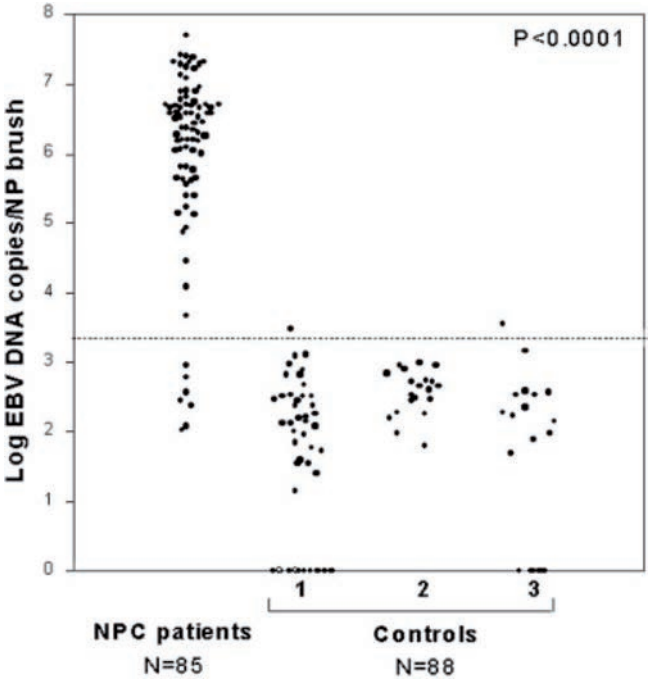

Figure 2 | Nasopharyngeal brushings obtained from NPC patients have significantly higher EBV DNA loads compared to non-NPC controls (P<0.0001).

Control group 1 consists of 45 healthy individuals (open circles indicate the 2 EBV-seronegative individuals), control group 2 of 22 patients with ENT complaints but without malignancy and control group 3 consists of 21 non-NPC tumour patients. The dotted line indicates the cut-off value, calculated as the mean EBV DNA load in NP brushings from the controls plus 3 standard deviations.

Cut-off values (COV) for EBV LightCycler were defined by calculating the mean EBV DNA load in NP brushings from the control population plus 2 standard deviations (i.e. 1666 EBV DNA copies/NP brushing) or the mean plus 3 standard deviations (i.e. 2312 EBV DNA copies/NP brushing). These COV were used to determine sensitivity, specificity, positive and negative predictive values, as indicated in table 1. Using either COV, these diagnostic parameters were ≥90%. Of the 79 individuals exceeding the COV in PCR, 77 were confirmed as NPC cases by EBER-positive biopsies (see table 1). Only 2 controls (one healthy EBV-carrier and one non-NPC tumour patient) showed a slightly elevated EBV DNA load above COV, illustrating the high specificity of this approach (Figure 2, table 1).

Table 1 | Specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) of EBV DNA load quantification in nasopharyngeal brushings at different cut-off values (COV).

	COV = Mean EBV load in controls + 2 standard deviations (= 1666 EBV DNA copies/brushing)		COV = Mean EBV load in controls + 3 standard deviations (= 2312 EBV DNA copies/brushing)	
	Below COV	Above COV	Below COV	Above COV
NPC patients (N=85)	8	77	8	77
Controls (N=88)	85	3	86	2
Specificity	97%		98%	
Sensitivity	91%		91%	
PPV	96%		97%	
NPV	91%		91%	

Interestingly, the healthy carrier showed IgA reactivity to EBNA1 and VCA-p18, which is very characteristic for NPC patients, but is normally rarely observed in healthy EBV-carriers[11], indicating putative aberrant EBV activity. We are now regularly examining this volunteer for EBV and clinical parameters. The EBV DNA load in the NP brush samples from NPC patients showed no relation to the number of cells, defining one cell as 2 diploid human genome equivalents (Spearman's rho = 0.29; see figure 3), indicating that most DNA collected on the brush was derived from normal cells. This is in agreement with the cytological observation revealing many polymorph nuclear leukocytes and normal columnar epithelial cells with only sporadic NPC tumour cells per field of microscopic observation (data not shown). Two EBV-negative healthy individuals yielded negative EBV DNA values whereas the cellular DNA load was within the normal range. A significant linear correlation was obtained between the EBV DNA load expressed per NP brush and the EBV DNA load expressed per human diploid genome equivalent (Spearman's rho = 0.94; $p<0.0001$), as shown in figure 3. This reflects the constant level and abundance of normal cells relative to NPC cells in the brush samples. Therefore the value of EBV DNA load per brush was used for all further analyses.

The amount of EBV DNA in the NP brushing was not related to TNM stage (Kruskal-Wallis test p=0.223) or primary tumour size (Kruskal-Wallis test p=0.755). Finally, EBV DNA load in the NP brushing was unrelated to EBV DNA load in the whole blood of patients (r^2=0.05; p=0.07), as determined by a recently described 99 bp LC-PCR[22].

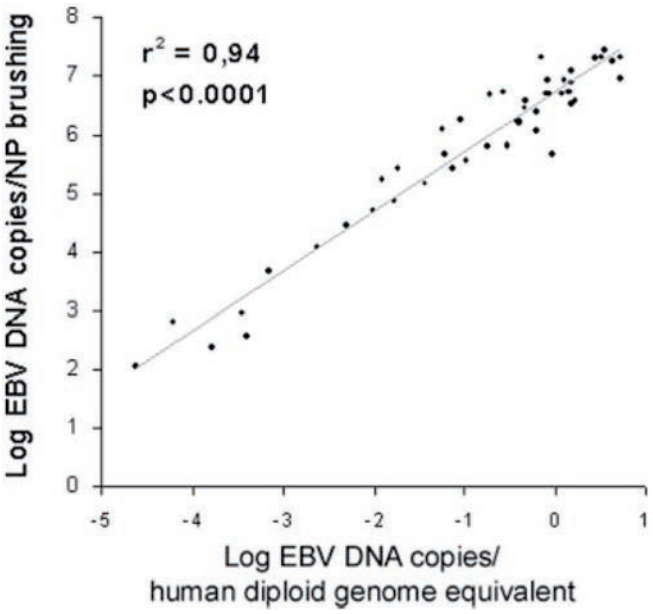

Figure 3 | Linear correlation between numbers of EBV DNA copies expressed per nasopharyngeal brushing sample ("EBV DNA load/NP brushing") or per human diploid genome equivalent.

EBV RNA is abundantly detectable in NP brushings: EBV is transcriptionally active and expresses the carcinoma-specific BARF1 oncogene

For a total of 78 out of 85 NP brushings samples (92%) from NPC patients, RNA profiling could be performed using NASBA and RT-PCR. As a control for RNA quality and in order to detect general EBV transcriptional activity, we performed a NASBA assay for EBNA1 mRNA, which is expressed in all EBV-associated malignancies, including NPC[12]. Sixty-seven out 78 (86%) were EBNA1 mRNA-positive, reflecting EBV transcriptional activity in NP brushing samples (see figure 4). This was confirmed by parallel RT-PCR positivity for the non-coding BARTs, which were detected in 69% of samples tested and in 76% of EBNA1 mRNA positive samples (table 2).

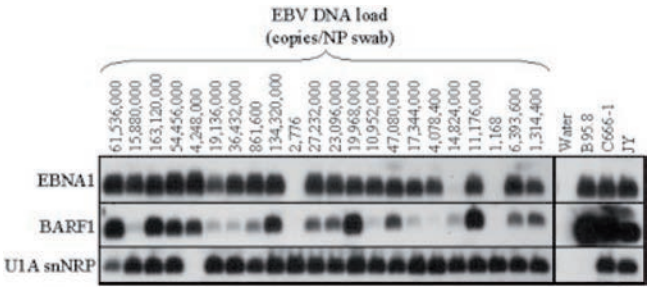

Figure 4 | EBNA1 and BARF1 RNA expression in NP brushings samples as determined by NASBA, in relation to EBV DNA load. Shown is an autoradiogram of NASBA products hybridised with a radioactive labelled internal oligonucleotide probe.

Table 2 | EBV RNA positivity in nasopharyngeal brushing samples obtained from NPC patients.

EBV RNA target	All brushing samples		Brushing samples stored at -80ºC	
	No. positive NP brushings/ no. tested	No. positive NP brushings/no. EBNA1-positive NP brushings	No. positive NP brushings/ no. tested	No. positive NP brushings/no. EBNA1-positive NP brushings
EBNA1	67/78 (86%)	na	28/32 (88%)	na
BARF1	58/78 (74%)	57/67 (85%)	28/32 (88%)	27/28 (96%)
BARTs	54/78 (69%)	51/67 (76%)	23/32 (72%)	20/28 (72%)
LMP2	67/78 (86%)	66/67 (99%)	29/32 (91%)	28/28 (100%)

Data are shown for all collected brushing samples for which RNA analyses could be completed (N=78, including samples stored at room temperature) and for the batch of fresh samples that was consistently stored at -80ºC until RNA isolation and amplification procedures (N=32).

EBNA1: Epstein-Barr nuclear antigen 1; BARF1: BamHI-A rightward frame 1; BARTs: BamHI-A rightward transcripts; LMP2: latent membrane protein 2

To confirm that the elevated EBV DNA loads in the NP brushings indeed reflect carcinoma-associated EBV activity, we investigated the expression of BARF1 mRNA, a carcinoma-specific viral transcript. BARF1 mRNA positivity was seen in 58/78 (74%) of all samples and in 57/67 (85%) of EBNA1 mRNA-positive samples. This carcinoma-specific EBV transcription in NP brushings directly reflects oncogenic viral activity at the anatomical site of tumour development.

In addition to BARF1 expression, we studied expression of LMP2 mRNA, which is consistently expressed in NPC[39]. LMP2 mRNA was detectable in 67/78 (86%) of NP brushings and in 66/67 (99%) of EBNA1-positive samples.

The first batch of NP brushing specimens analysed for EBV mRNA arrived at ambient temperature at the Amsterdam laboratory (see Materials and Methods), which may influence RNA quality. Therefore we also separately analysed the first and second (frozen) batch of samples. For the first batch EBNA1 and BARF1 mRNA positivity was respectively 39/46 (85%) and 30/46 (65%) in brushing samples from NPC patients. The second batch (N=32) showed significantly higher mRNA positivity (28/32 (88%) for EBNA1 and 28/32 (88%) for BARF1 mRNA in NPC patients, indicating an effect of shipment conditions on RNA quality.

Remarkably, the 8 NP brushing specimens from NPC patients that were below COV in EBV DNA load assay (see table 1; EBV DNA load range 108-910 EBV DNA copies/NP brushing), were also negative for EBNA1, BARF1, BARTs and LMP2 RNA, probably reflecting incorrect sampling. Beta-globin DNA copies were within the normal range for these samples.

In the control population, no EBV mRNA was detectable in the tested NP brushings, despite positivity for a low copy cellular U1A snRNP housekeeping RNA and the presence of EBV DNA. This confirms our previous findings that BARF1 mRNA is a carcinoma-specific marker[10,12,15].

DISCUSSION

Diagnosis of primary NPC currently requires pathological assessment of a biopsy taken from the post-nasal space. This is an invasive and painful procedure that may lead to extensive bleeding and cannot be repeated easily without compromising the patient. In this study, we show that detection of EBV DNA load and carcinoma-specific BARF1 mRNA in NP brushings provides a highly specific and non-invasive diagnostic tool for NPC. Of the 79 individuals that were above COV in the quantitative EBV PCR assay, 77 were subsequently diagnosed with NPC by examination of a nasopharyngeal biopsy and demonstration of EBV involvement by *in situ* EBER RNA expression. Although detection of molecular markers may not entirely replace the biopsy, as previously indicated[40,41], EBV DNA quantification in NP brushings combined with qualitative BARF1 and EBNA1 mRNA detection may greatly reduce the number of invasive nasopharyngeal biopsies required. In addition, this method may be used repeatedly for post-therapy monitoring and detection of local recurrent disease (study in progress). The feasibility of this approach was recently shown[27,42] and our ongoing pilot study confirms the observations that high post-therapy EBV DNA loads in brushing samples correlate with unfavourable clinical outcome (data not shown). Our preliminary data furthermore illustrate that follow-up NP brushing sampling is a non-invasive and well tolerated-prognostic strategy, without any noticeable side effects and suited for monitoring of kinetic changes in EBV DNA load during chemo-and radiotherapy..

The EBV DNA load approach has high positive and negative predictive value (see table 1). It can assist in clinical patient management and it can be repeated more easily and frequently than the biopsy. NP brushing with direct storage of samples in the described DNA and RNA-stabilizing buffer[33,34] is a relatively cheap method that, combined with a portable nasendoscope, may be used "in the field" in regions with high NPC incidence, e.g. in developing countries such as Indonesia, where medical facilities are poor. Nasendoscopy may even not be required for experienced examiners. Thus, the strategy described here may improve future population-based screening studies for confirmation of NPC presence, for example following initial risk assessment by serological screening for EBV-specific IgA and IgG[11]. Monitoring of EBV parameters reflecting increased viral burden and carcinoma-specific transcriptional activity is more sensitive and specific than previously described cytological evaluation of NP brushing smears[43,44]; Harijadi et al, unpublished data).

A small number of NP brushing samples was below COV in LC-PCR. These specimens were also negative for EBNA1 and BARF1 transcription, despite high total cell numbers in the sample as determined by quantitative human globin PCR. This may be due to erroneous brushing of an anatomical site with no NPC involvement or limited release of NPC cells during brushing. NPC cells at the surface may also be obscured by blood or tumour detritus[43]. However in our study we did not find any correlation between NP bleeding and EBV DNA levels (data not shown). Moreover, there is a possibility that a deeply located tumour is missed by the NP brushing procedure, while the deep biopsy may be able to yield sufficient number of tumour cells in such case[27,43]. It is not uncommon that multiple biopsies are needed in some patients to make definite NPC diagnosis[25]. The NP brushing procedure has a false negativity rate comparable to the diagnostic nasopharyngeal biopsy. Repeated brushing is feasible because of its non-invasive nature compared to nasopharyngeal biopsies. Whether repeated brushing could give additional primary diagnostic value in individuals below EBV DNA load COV could not be assessed in our current retrospective EBV DNA load analysis, but the feasibility of repeating the brushing procedure without significantly compromising the patient is apparent from the ongoing prognostic follow-up study discussed above.

Some studies showed complete absence of EBV DNA in NP brushings obtained from healthy EBV-carriers[24,26]. This may be due to insensitive qualitative (multiplex) PCR assays[24] or insufficient brushing of the nasopharyngeal epithelium in these controls. However, several other studies[25,45] and our present study using sensitive quantitative real-time PCR, have found EBV DNA in brushing specimens from most healthy carriers, albeit at low levels. This is not surprising, as EBV-infected B-lymphocytes have a homing preference for the nasopharyngeal region (Waldeyer's ring) and virus is shed into the oropharyngeal space[46,47].

It is yet unclear whether the carcinoma-specific BARF1 mRNA is consistently expressed in all NPC cases. Decaussin[48] et al showed BARF1 mRNA expression in 85% (23/27) of EBV-positive, North African NPC biopsies (all confirmed by protein detection). In our current larger study we a found a similar positivity rate, with BARF1 mRNA in 85% of EBNA1 mRNA-positive samples. This may indicate that BARF1 expression is heterogeneous between tumours, although low expression levels in a minority of tumour cells cannot be excluded. We could detect viral LMP2 mRNA in nearly all tested samples and recent studies showed that this gene may contribute to carcinogenesis[49,50]. We believe BARF1 mRNA is a better NPC marker, because LMP2 may be detectable in latently EBV-infected B-cells in the circulation of healthy EBV-seropositive individuals and is therefore not carcinoma-specific[46]. However, since LMP2 mRNA is absent from control brushings, the high transcription level in tumour cells in NPC patients may be used as carcinoma marker, e.g. by using a quantitative NASBA or RT-PCR assay.

In addition to viral RNA profiling, the NP brushing specimen could also be useful for investigating EBV gene polymorphisms, mutations in human oncogenes, epigenetic changes and for cellular gene expression profiling to predict tumour behaviour and prognostication. Sensitivity and specificity of the NP brushing procedure may be increased by using a combination of molecular carcinoma markers[25,40,41]. Cellular genetic markers, e.g. hypermethylation of numerous tumour suppressor gene promotor regions, have been described[25,40,41] and could putatively be added to future multi-analyte NPC screenings approaches, for example to confirm first-round serological screening. This is, however, the first description of an EBV-encoded oncogene, i.e. BARF1, as a carcinoma marker in NP brushings.

Although EBV DNA load monitoring in blood, plasma or serum of NPC patients has widely been described, circulating viral DNA levels are relatively low (100-2,000 copies/ml plasma or blood) in a significant subset of NPC patients thus making accurate quantification and definite diagnosis difficult[22]. The circulating EBV DNA is fragmented and is probably derived from apoptosed NPC cells releasing their DNA content into the blood[22,23]. Furthermore, blood samples from NPC patients are BARF1 mRNA-negative[22], indicating absence of circulating tumour cells. Thus demonstration of an elevated viral DNA burden plus carcinoma-specific viral mRNA expression in NP brushings is preferable in patients suspected for NPC In conclusion, quantitative monitoring of EBV DNA and simultaneous EBNA1 and BARF1 mRNA detection in NP brushing samples is a specific, non-invasive tool for diagnosis in patients suspected for NPC, directly detecting aberrant and carcinoma-specific EBV activity at the anatomical site of primary tumour development. Due to its non-invasive nature the NP brushing method would be a valuable tool that can be used frequently during prognostic

follow-up. Moreover, the NP-brush method may be particularly useful as confirmatory test for NPC risk assessment in population screening studies, using for example IgA and IgG serology to specific EBV proteins as initial diagnostic marker[11]. Our approach may further support implementation of population-based screening programs in South-East Asia and North Africa, were NPC incidence is the highest. The feasibility of such an approach is underlined by studies from our institute indicating detection of human papillomavirus DNA in cervical brushings as a sensitive tool for population-based cervical carcinoma screenings programs[52,53].

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Chapter 4

Epstein-Barr virus DNA load in nasopharyngeal brushings and whole blood in nasopharyngeal carcinoma patients before and after treatment

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ABSTRACT

Purpose: Nasopharyngeal carcinoma (NPC) is consistently associated with Epstein-Barr virus (EBV) and highly prevalent in Indonesia. EBV-DNA load can be used for early diagnosis and may have prognostic value. In this study EBV-DNA load was evaluated in minimal invasive nasopharyngeal (NP) brushings and whole blood for initial diagnosis and therapy assessment against the standard of care diagnosis by biopsy with EBV-RISH and standard EBV-IgA serology.

Experimental Design: NP-brushings and blood samples were collected from 289 consecutive ENT patients suspected of NPC and 53 local healthy controls. EBV-DNA load was quantified by real-time PCR and serology by peptide-based EBV-IgA ELISA. Tissue biopsies were examined by routine histochemistry and by EBER RNA in situ hybridization.

Results: Repeated NP brushing was well tolerated by patients and revealed high viral load in the 228 NPC cases at diagnosis compared to 61 non-NPC cancer cases and healthy controls ($p<0.001$). The diagnostic value of EBV-DNA load in blood and EBV-IgA serology was inferior to the NP brush results. The level of EBV-DNA load in brushes of NPC patients was not related to T, N or M stage, whereas elevated EBV-DNA load in blood correlated with N and M stage. EBV DNA levels in brushings and whole blood showed a significant reduction at 2 month post-treatment ($p=0.001$ and $p=0.005$, respectively), which was not reflected in EBV-IgA serology.

Conclusions: NP brush sampling combined with EBV-DNA load analysis is a minimal invasive and well-tolerated diagnostic procedure, suited for initial diagnosis and follow-up monitoring of NPC.

TRANSLATIONAL RELEVANCE

Diagnosis and post-treatment monitoring of Epstein-Barr virus (EBV) associated nasopharyngeal carcinoma (NPC) is complicated and requires repeated painful biopsies and pathological examination. Early tumor detection and timely initiation of treatment are important for patient survival. The results from this study in 228 NPC patients reveal that simple non-invasive nasopharyngeal (NP) brushing plus EBV-DNA load as tumor marker gives excellent diagnostic and prognostic results compared to the biopsy. The NP-brush approach proved better than EBV-DNA load assessment in blood and EBV-IgA serology. The data suggests that NP-brush sampling may provide a useful instrument for direct in situ NPC tumor detection in populations with symptoms suspected of NPC and may replace repeated biopsies during follow-up. The NP-brush is not perceived as painful by patients, is suited for remote sampling in regional hospitals and allows parallel assessment of additional tumor markers. The NP-brush appears well suited for use in NPC screening in high incidence regions, like Indonesia.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a distinct head & neck cancer, occurring at high frequency in Southeast-Asian, North-African and Inuit populations[1]. In Indonesia, with an ethnically diverse population of 225 million people, NPC is the most common head and neck cancer with high prevalence among native populations and an overall incidence estimated at 6.2/100,000[2]. In the Dr. Cipto Mangunkusumo Hospital, Jakarta, NPC is the 5th most frequent cancer overall after cervical carcinoma, breast cancer, colon and skin cancer with an incidence of 6.6% (cervical cancer 16.1%, breast cancer 14.5%, colorectal cancer 9.9%). NPC is the most common tumor in the head and neck, constituting 23.8% of all head and neck cancer cases[3].

Because NPC is highly radiosensitive the mainstay treatment is radiotherapy (RT), which can result in a 5-year overall survival of 90% for early stage disease I and in late stage disease (stage 3 and 4) the treatment outcome has a cure rate of less than 58%[4]. Thus, diagnosis at early stage of NPC is a clear medical need. Unfortunately more than 85% of NPC patients in Indonesia present in the clinic with advanced stage of disease and treatment outcome is poor[3].

NPC has a close association with Epstein Barr virus (EBV), a ubiquitous human herpesvirus infecting over 90% of the world population and viral gene products are expressed in all tumor cells. EBV is present in almost 100% of undifferentiated NPC cases (UCNT WHO type III), whereas its association with squamous cell carcinoma (WHO type I) and non-keratinizing carcinoma (WHO type II) is variable. In NPC endemic regions WHO type I and II tumors are also frequently associated with EBV[5], but in non-endemic regions these often result from tobacco and alcohol abuse[6]. Undifferentiated NPC represents 85% of all NPC cases in endemic regions and is a major cause of cancer morbidity and mortality imposing a significant socio-economic burden to families and the population in general[7].

Currently, diagnosis of NPC requires a biopsy from the suspected tumor site with histopathological assessment and demonstration of EBV involvement by *in situ* hybridization for EBER1/2 RNA or immunohistochemistry for EBNA1 or LMP1 protein. The detection of EBER transcripts by *in situ* hybridization remains the standard of care for identifying latent EBV infection. A biopsy from the post-nasal space is an invasive and painful procedure that may lead to extensive bleeding and cannot be repeated easily without compromising the patient[8]. At early stage NPC often presents with minimal or nonspecific local symptoms and the nasopharynx is difficult to access for (repeated) routine examination making early diagnosis challenging. Thus the biopsy is crucial for defining NPC as cause of symptoms and subsequent medical handling. In addition, it is important to obtain biopsies of adequate depth as nasopharyngeal carcinoma may spread submucosally and are easily missed by endoscopic examination, even in patients with an obvious exophytic tumour, due to slough,

necrotic tissue, and inflammatory tissue overlying the tumor. Therefore biopsy with a small endoscopic forceps may result in a high false negative rate. A representative biopsy can be difficult to obtain and requires the use of flexible and rigid endoscopes to allow good visualization of the nasopharynx. Local anesthesia, permit biopsies to be taken under direct vision and therefore anesthesia is recommended to avoid missing small or submucosal lesions yielding sensitivity of 95.1% and 95.6% respectively[9,10]. When no obvious tumor is present a biopsy from the lateral pharyngeal recess can be performed because this is the most common site for early disease[11].

There is a clear need for more simple non-invasive diagnostic assays for early NPC detection, in particular in endemic regions, which can also be used in monitoring therapy requiring repeated sampling. Previous studies revealed nasopharyngeal (NP) brushing as a simple procedure with minor discomfort, being well tolerated and reflecting carcinoma-specific EBV involvement at the anatomical site of tumor development, thereby reducing the need for invasive biopsies[12-14]. This procedure has promise as confirmation test in serological NPC screening programs and has potential as prognostic tool for therapy assessment and follow-up monitoring. Furthermore, aberrant tumor-associated DNA methylation patterns can be analyzed in the same brush specimen[15,16]. In addition to viral load in NP brushings, measuring the level of EBV DNA in whole blood, plasma or serum of NPC patients before and after treatment may be valuable for assessment of disease progression[17], since levels of EBV DNA in the circulation of NPC patients with recurrence were shown to be much higher than EBV DNA levels of those who remain in continuous clinical remission[18,19]. These studies indicated that monitoring EBV DNA load may provide useful diagnostic information for NPC diagnosis and post-treatment management.

The present study evaluates the diagnostic and post-treatment value of viral DNA load measurement in minimal invasive NP brushings and in parallel in whole blood samples collected at diagnosis and 2 months after start of therapy in 228 patients with advanced NPC. The viral load was compared to standardized peptide-based EBV-IgA serology and clinical treatment response.

MATERIAL AND METHODS

Patients and controls

Two hundred and eighty nine consecutive patients presenting to the ENT clinic of Dr. Cipto Mangunkusumo Hospital, Universitas Indonesia in Jakarta with suspected NPC during 2006-2009 were enrolled into this study. About 20% of the patients were referred by regional health centers where initial diagnosis was performed. Medical ethical approval

for this study was obtained and all patients and controls signed for informed consent. TNM staging was done for all patients using the 2002 American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) staging system. Assessment for diagnosis included: medical history, particularly on NPC related symptoms, physical examination for enlarged neck node and examination of the suspected nasopharyngeal (NP) lesion by fiber-optic nasopharyngoscopy with photography and CT scans. In all patients a nasopharyngoscopy guided NP brushing was performed first, followed by biopsy from the same area of the suspected NPC. Endoscopic findings were classified as normal (no tumor), suspicious tumor, or clearly abnormal. Of the 289 patients at intake, 228 had biopsy proven NPC and 61 were proven to have a variety of malignant and non-malignant head and neck diseases and served as clinical controls in this study, as specified in Table 1. Unfortunately due to problems inherent to the Indonesian health care system (lack of medical facilities, low social economic status, insufficient insurance coverage and the often remote area's where patients are living) detailed follow-up proved difficult. A total of 202 brushings, 149 whole blood and 174 serum samples at diagnosis and from follow-up 69 brushings, 65 whole blood and 68 serum samples were available for analysis (Table 2). Clinical characteristics and NPC stage information is given in Table 3. Diagnosis was based on routine pathological assessment of paraffin-embedded tumor biopsy specimens and WHO typing of NPC was assessed by 2 independent pathologist. The presence of EBV was confirmed by EBER-RISH using the commercial PNA-based hybridization kit (Dakocytomation, Glostrup, Denmark) in 116 of 228 patients from whom an adequate biopsy specimen was available.

Table 1 | Numbers of patients and controls used for validation of viral load by NP brush

Patient description			
NPC			228
EBV-related malignancy			19
	Non–Hodgkin lymphoma	8	
	T/NK cell lymphoma	10	
	Burkitt's lymphoma	1	
Non NPC head and neck carcinoma			25
Other ENT disorder			17
Healthy control			53

Table 2 | Numbers of samples of NPC patients at diagnosis and 2 month follow-up

Patients with NPC	**NP brushing**	**whole blood**	**serology**
At diagnosis	208	149	174
After 2 months follow-up	69	65	68

Table 3 | Characteristics of NPC Patients (n= 228)

		Number	Percentage (%)
Sex	Male	164	71.9
	Female	64	28.1
Histopathology	WHO 1	28	12.3
	WHO 2	5	2.2
	WHO 3	195	85.5
Age	<10	5	2.2
	10-20	19	8.3
	21-40	81	35.5
	>=41	123	53.9
T Stage	T1	18	6.4
	T2a	16	5.7
	T2b	69	24.6
	T3	53	18.9
	T4	72	25.6
N stage	N0	25	11
	N1	61	26.8
	N2	48	21.1
	N3a	77	33.8
	N3b	17	7.5
N Stage	N0	25	11
	N+	203	89
M Stage	M0	210	74.7
	M+	18	6.4
Stage AJCC-UICC	Stage I	2	0.9
	Stage IIA	1	0.4
	Stage IIB	25	11
	Stage III	55	24.1
	Stage IVA	42	18.4
	Stage IVB	85	37.3
	Stage IVC	18	7.9
Stage summary	Early	3	1.3
	Advance	225	98.7
Type treatment	Neoajuvant+ RT	81	35.5
	Neoajuvant+ HPF	40	17.5
	Neoajuvant + CRT	9	3.9
	Concurrent CRT	87	38.2
	Radiotherapy	1	0.4
	Chemotherapy full dose	9	3.9
	No treatment	1	0.4
Clinical Response treatment at 2 months post treatment	Complete response	52	22.8
	Partial responses	30	13.2
	Progressive disease	2	0.9
	Death	7	3.1
	Loss to FU	137	0.9

RT: Radiotherapy, HPF: Hyperfractination, CRT: Chemoradiation, FU: Follow up

Treatment

In NPC cases radiotherapy was uniformly administered to the primary tumor and neck region. The total dose delivered was 66 to 70 Gy during 6 to 8 weeks by conventional fractionation or hyperfractionation accelerated radiotherapy. Neoadjuvant/adjuvant chemotherapy consisted of 5-FU (1000 mg/m^2 day 1-5) and cisplatin (100 mg/m^2 day 1) in 3 cycles every 3 weeks. Concurrent chemotherapy was delivered with cisplatin at 40 mg/m^2 weekly during radiotherapy courses. Due to under-capacity of radiotherapy and the poor financial situation of most patients optimal treatment, i.e. full chemoradiation, was not always feasible and different treatment protocols had to be implemented.

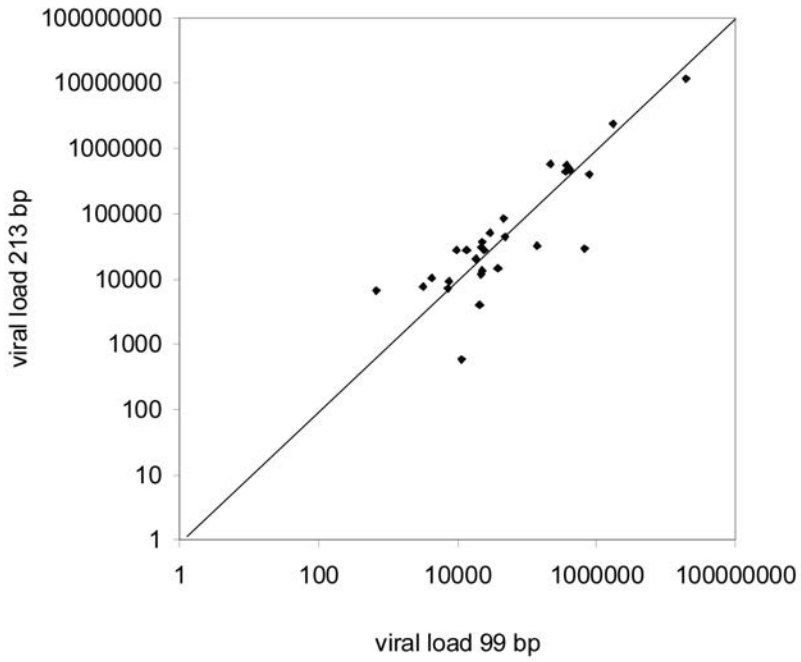
Sampling procedures

NP brushing was performed under rigid or flexible endoscopic guidance by experienced ENT specialists and ENT resident trainees. Endoscope-guided NP brushings were performed under local anesthesia (1% Lidocaine spray, Astra Zeneca, Waltham, USA). An endoscope was used to evaluate the entire nasopharynx and photographs were taken routinely from the site of tumor involvement. Localization and appearance of the tumor was defined and graded into 3 groups (none, suspicious and clear abnormal). A Cytobrush Plus (Medscand, Malmo, Sweden) was employed in combination with a plastic catheter covering the entire brush to prevent contamination by cells from non- NP sites. The catheter covering the cytobrush was inserted via the nose until the NP cavity was reached. Subsequently, the brush was released from the catheter and the cytobrush was rotated several times over the NP epithelium at the site of the suspected lesion, returned into the catheter and removed. Immediately after sampling the brush tip (1.5 cm) was cut and placed in 4 ml of NucliSens Lysis buffer (LB) (BioMerieux, Marcy l'Etoile, France) mixed well and stored in 1 ml aliquots at -80^{0}C until use[14,20]. In all NPC suspected patients NP brushings were obtained from the site of suspected tumor involvement before taking the biopsy at the same site. In 20 patients both sides of the NP wall were brushed at diagnosis or during follow-up under endoscopic guidance (twenty-five 2-sided samples were collected). To compare the level of discomfort between the brushing procedure and the biopsy, 57 patients at random answered a questioner-form based on visual analog scale 1-10. Furthermore we performed standard nasopharyngoscopy and brushings with informed consent in 53 healthy regional controls,. At the same time 5 ml whole blood was taken of which 4.5 ml was used to make serum for serology and 0.5 ml was added to 4.5 ml LB for measuring EBV-DNA load, exactly as described before[21,22]. Frozen samples were shipped on dry-ice and analyzed blindly to the NPC status for EBV-DNA load at the department of Pathology, VU University Medical Centre, Amsterdam, the Netherlands.

Quantification of EBV DNA load and cellular DNA by LightCycler-based real-time PCR assays

DNA was isolated from 1 ml NP brush samples in LB by silica-based nucleic acid extraction and eluted in 100 µl H_2O, exactly as described before[14,22]. Reagents for the isolation procedure were obtained from BioMerieux. EBV real-time PCR described for NP-brush samples in this study was based on amplification of well-conserved 213-bp region of the BKRF1 gene encoding Epstein-Barr nuclear antigen-1 (EBNA-1), a single-copy gene of EBV and blood samples were analyzed by PCR using a 99-bp region from the same EBNA1 region in order to reliably detect fragmented EBV-DNA, as described before[21,22]. Most brush samples were analyzed by both PCR assays, yielding no significant different result (Supplementary figure 1). Primers, probes and PCR conditions have been described in detail previously[14,22]. Cut-off value (COV) for EBV DNA load in NP brushings was defined at 2300 copies/brush, being the mean + 3xSD of brush EBV-DNA load in non-NPC case-controls as previously defined[14] and confirmed in the current group of healthy Jakarta EBV carriers, excluding 4 individuals with elevated EBV-DNA load also having aberrant EBV-serology, possibly relating to stress-induced EBV reactivation. The COV for EBV-DNA in blood was defined at 2000 copies/ml, based on prior studies[21,22]. These COVs were validated and confirmed in the healthy control group in this study used to determine sensitivity and specificity, positive and negative predictive values.

The amount of human diploid genome equivalent in NP brushing specimens was determined by quantitative LC-PCR targeting a 197 bp fragment of the human ß-globin gene[23].



Supplementary figure 1 | Comparison of the 99bp and 213bp PCR for measuring the EBV DNA load in NP brushings. Both primer pairs gave similar results

EBV serology

Serum samples from NPC patients, control patients and healthy controls (Table 2) were analyzed for IgA antibodies to EBV-specific immunodominant epitopes of VCA-p18 and EBNA1 using individual synthetic peptide-based ELISA assays for each marker exactly as described previously[24].

Statistics

One-way ANOVA was used for comparison of EBV-DNA load and EBV IgA antibody levels between NPC and non-NPC groups In addition one-way ANOVA was used for comparing EBV DNA load and antibody levels to TNM stage of NPC at intake. A p-value of <0.05 was considered to be significant.

Mann Whitney test :$p<0.001$ used for subjective evaluation for visual analog scale (VAS) between brushing and biopsy procedures to examine the median difference between two group (procedures) and for analysing the level of comfort of the performing a NP brush or biopsy.

The evaluation of viral DNA load of bilateral side nasopharyngeal brushing was performed by a Mann Whitney test. Testing the Viral DNA load decreases in NP brush and whole blood at diagnosis and after treatment of the paired samples was performed by a Wilcoxon test.

RESULTS

Patient characteristics

For this study 289 consecutive patients with suspected NPC were enrolled. In 228 cases NPC diagnosis was confirmed by pathological examination of the biopsy using routine histochemistry. Patient characteristics are summarized in Table 3. The non-NPC group consisted of patients diagnosed with EBV related malignancy, EBV negative non-NPC head and neck cancer, non-malignant ENT disorders and 53 healthy individuals (Table 1).

In the NPC group male-female ratio was 3:1 and 85% were classified as WHO type 3. Although the age of the majority of NPC patients (54%) was above 40 years, 11% was of juvenile (5-20 years) age. At presentation 99% of patients had advanced stage of disease, with 85 patients (37%) in stage IVB (AJCC-UICC staging system) and 18 patients (8%) had distant metastasis. The treatment of choice for these patients is a combination of chemotherapy and radiotherapy (Table 1). The patients with distant metastasis were treated with palliative chemotherapy For this study, 208 NP-brushes, 149 whole blood and 174 serology samples could be evaluated at diagnosis. Post-treatment NP brush samples of 69 patients were analyzed as well as 65 parallel whole blood (WB) and 68 serology samples.

Viral DNA load in nasopharyngeal (NP) brushings at diagnosis

An accurate well-validated real-time PCR procedure for EBV-DNA quantification , detecting a conserved region of the single copy EBNA1 (BKRF1) gene, was used for analyzing the EBV-DNA load in NP brushings taken at diagnosis. Clinical cut-off value (COV) for viral DNA load in NP brushings was previously defined at 2300 EBV DNA copies/NP brushing in healthy EBV seropositive individuals and non-NPC patients with various head and neck complaints[14] and was here confirmed in the healthy controls in the Jakarta population (Figure 1A). This COV was used to determine sensitivity, specificity, positive and negative predictive values of 94%, 90%, 84% and 80% respectively, as indicated in Table 4.

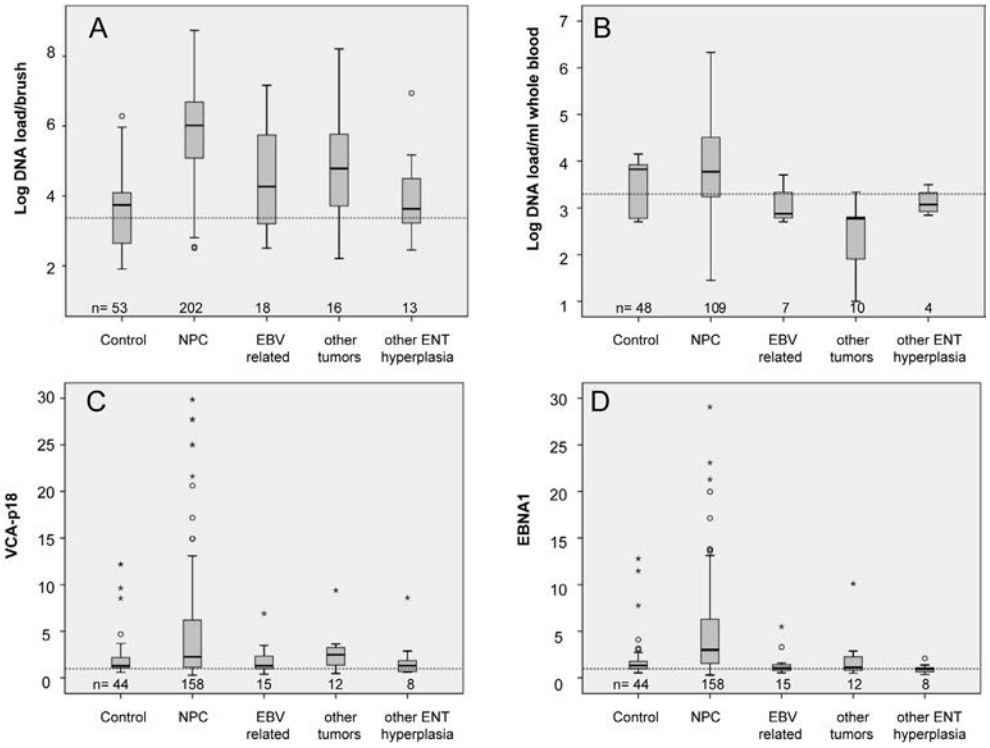


Figure 1 | EBV parameters at diagnosis.

A: Viral DNA load in NP brushings (*log scale*). The EBV-DNA levels observed were significantly different between NPC and healthy controls EBV-related malignancies (p<0.001), and other ENT disorders (p<0.001), whereas a near significant difference was found between NPC and non-NPC head & neck cancer (p=0.059). **B: viral DNA load in whole blood (*ilog scale*)**. The mean EBV DNA load in blood was not significantly different between NPC and healthy controls (p=0.601), EBV-related malignancies (p=0.109), and other ENT disorders (p=0.401), whereas NPC and non-NPC head and neck cancer did show a significance difference (p<0.001). **C: IgA VCA-p18 serology**. EBV-specific VCA-p18 IgA serology was significantly higher in NPC versus healthy controls (p=0.011), but not between NPC and EBV-related malignancy (p=0.21), non-NPC head and neck cancer (p=0.75), and other ENT disorders (p=0.57). **D: EBNA1-IgA serology.** EBNA1-IgA serology was significantly higher in NPC versus healthy controls (p<0.001), and EBV-related malignancies (p=0.018), and was close to significance for NPC versus non-NPC head and neck cancer (p=0.20), and other ENT disorders (p=0.054). The dotted line in each graph represents the cut-off value for each assay, as defined in the methods section.

Table 4 | Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of EBV markers

	Brush	Whole Blood	IgA VCA P18	IgA EBNA 1
Sensitivity	94.3	71.1	65.4	74.3
Specificity	90.0	50.0	60.0	72.0
PPV	84.4	85.7	84.7	89.8
NPV	80.0	20.0	33.3	44.6

Figure 1A shows that NP brushings from NPC patients showed significant higher levels of viral load compared to controls (median NPC $1.0x10^6$, range 0-$1.9x10^8$ versus median $4.0x10^3$, range 0-$1.2x10^5$, $p<0.0001$).

EBV-DNA was detected above COV in 95% of NPC cases, confirming NPC tumor cell presence. The higher EBV DNA values in NP brushings in the 'other' tumor group compared to the EBV related tumors may be explained by the location of the tumor in the ENT region which might associate with reactivating EBV. The non-NPC EBV associated tumors are not located in the ENT region. Frequently extreme EBV-DNA levels were reached in NPC cases, up to 100 million copies of EBV-DNA per brush. Ten cases (5%) had an EBV-DNA level below COV. Viral DNA load at diagnosis was not related to age or sex of NPC patients (data not shown).

Erroneous sampling was excluded by quantifying the cellular beta-globin DNA which showed similar host genomic levels (3-10 million copies/brush), indicating that brush sampling itself was done appropriately[14]. However, absence of EBV load may be caused by sampling outside the tumor field. Brush viral DNA load in NPC cases was higher than in patients with non-NPC head and neck cancers ($p=0.059$), other EBV related malignancies ($p=0.001$) and non-malignant ENT complaints ($p<0.001$). However, EBV-DNA load in NP-brushings of these patients with mainly advanced stage NPC did not correlate with T- N- or M- substage of the tumor at diagnosis, as shown in Figure 2A, C and E (p-values of 0.60, 0.071 and 0.092, respectively, as determined by one-way ANOVA). Some control individuals having no detectable NPC tumor mass did show elevated EBV-DNA levels. In these cases EBV IgA serology was also elevated indicating EBV reactivation (data not shown), as recently found in defined NPC risk groups in Indonesia[25].

Viral DNA load in whole blood at diagnosis

The whole blood EBV-DNA load of NPC patients at diagnosis was significantly higher than the clinical COV of 2000 copies/ml whole blood[21] compared to the control groups and even compared to that in other EBV related malignancies (Figure 1B). However and importantly,

a high number of NPC cases had low (<COV) or undetectable EBV-DNA levels in blood which was even observed in some patients with bulky disease (stage IVA and IVB), confirming our previous independent findings[21]. No correlation was found between EBV-DNA load in whole blood and T-stage of the tumor at presentation as shown in Figure 2B (p=0.25). However, considering the positive samples only, a correlation was found between whole blood EBV-DNA load and N and M stage, (p<0.001 and p=0.010, respectively, Figure 2D and F).

Serology IgA VCA-p18 and IgA-EBNA1

IgA VCA-p18 serology, reflecting viral replication at the mucosal surface, showed higher values in sera from NPC patients at primary intake (median 2.3, range 0.29-30), compared to healthy controls (p=0.001). Sera form 79.8% of the patients with NPC had IgA VCA-p18 values above the COV level. The sera obtained from all other patient groups including the EBV related malignancies and non-NPC head & neck cancer had lower antibody levels against VCA-p18 compared to NPC patients and did not reveal further statistically significant differences between the groups. (Figure 1C)

IgA EBNA1 serology, reflecting latent (tumor) antigen expression, revealed significant higher values in NPC cases compared to other groups resulting in 85.6% of the patients having IgA EBNA1 responses above the COV. NPC patients have higher median value (median 3.0, range 0.3-29) compared to EBV related malignancy (median 1.1, range 0.53-5.5), other malignant conditions (3.05, range 0.3-29), healthy controls, (median 1.1, range 0.5-4.1), and other ENT disorders (median 1.1, range 0.5-10), (p<0.05) (Figure 1D). No correlation was found between VCA-IgA or EBNA1-IgA antibody levels in ELISA and TNM staging of the NPC tumor at intake (data not shown).

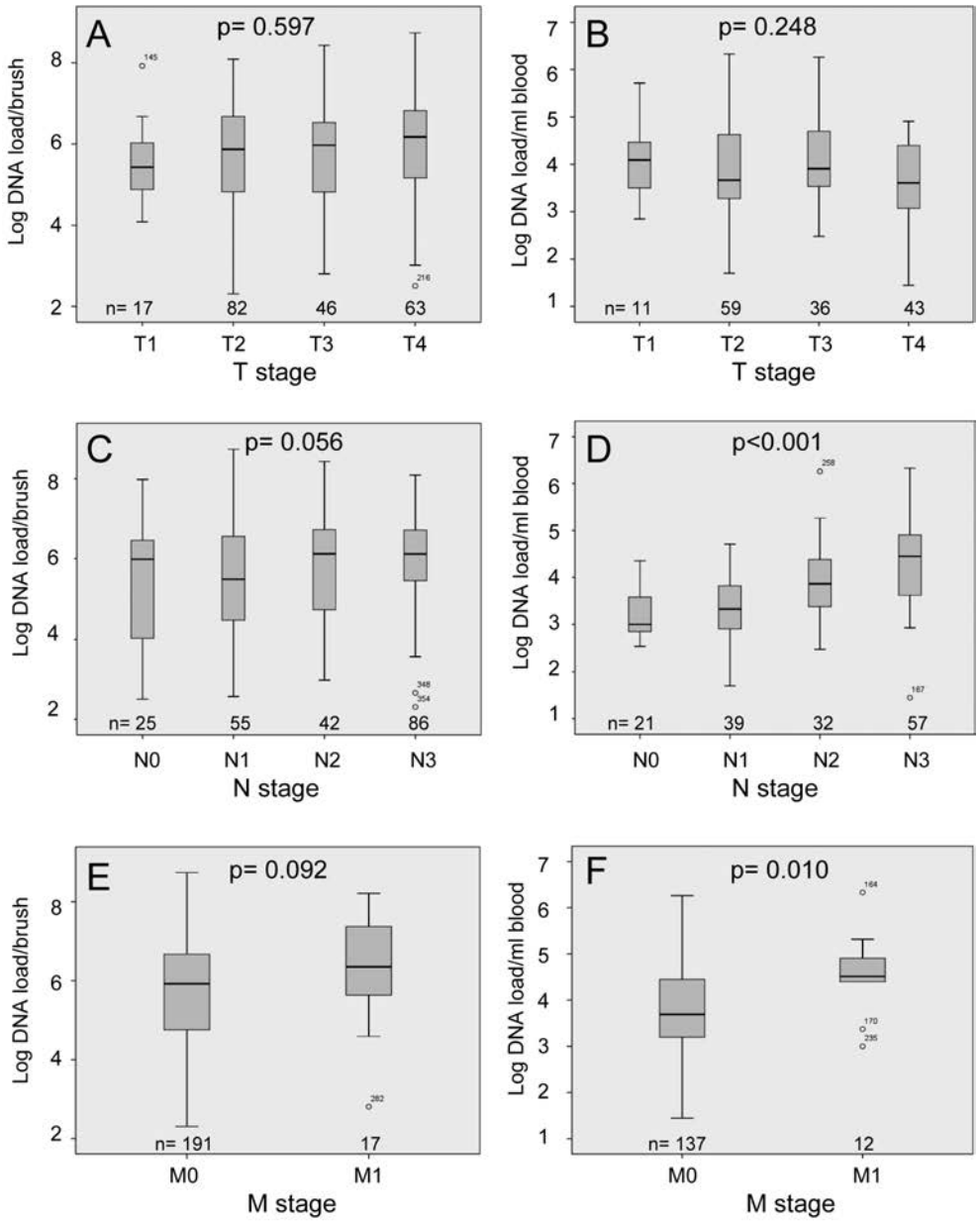


Figure 2 | EBV markers in relation to tumor characteristics at intake

A: Correlation of EBV-DNA load in brush with T stage (DNA copies in log scale), showing no relation using Anova (p=0.597). B: Correlation of EBV-DNA load in whole blood (WB) with T stage (p=0.248), C: Correlation of EBV-DNA load in brush with N stage (p=0.056), D: Correlation of EBV- DNA load in WB with N stage (p<0.001), E: Correlation of EBV-DNA load in brush with M stage (p=0.092),. F: Correlation of EBV-DNA load in WB with M stage (p=0.010).

Diagnosis by biopsy versus brushing

Biopsy was performed as standard of care diagnosis in all 228 NPC patients. We obtained information on the level of discomfort experienced during brushing and biopsy procedures in 57 patients, which were quantified by Visual Analogue Scale (VAS). The brush procedure was characterized by a median VAS score of 5 (range 3-6), which is significantly less compared to the biopsy with a median VAS of 9 (range 4-10; Kolmogorov Smirnov: $p<0.001$). Only 1 patient stated the biopsy was less painful than brushing.

In 11 patients repeated biopsies were required to obtain the diagnosis. One patient needed even three subsequent biopsies to obtain diagnostic evidence explaining the mass observed by CT Scan. In all 11 cases the viral DNA load in the initial brush was above COV allowing direct diagnosis.

In a selected group of 25 patients giving separate informed consent, we collected NP-brushings from both sides of the nasopharyngeal cavity, i.e. at and opposite to the suspected tumor site (defined by location of neck node in most cases). EBV DNA load values in parallel brushings were higher at the tumor site (72% >COV; median 16.700 c/brush; mean 188.782 c/brush; range 414 - $4.7x10^6$ copies/brush) compared to the opposite site (48% >COV; median 2.400 c/brush, mean 43.258 copies/brush; range 0 - $1.1x10^6$ copies/brush) (Figure 3). These differences were not statistically different ($p=0.13$). However, in NPC cases both the median and mean EBV DNA level in brushings taken from the non-lesional side of the nasopharynx were still significantly higher that the EBV DNA load observed in non-NPC tumors and ENT-hyperplasia ($p<0.001$)

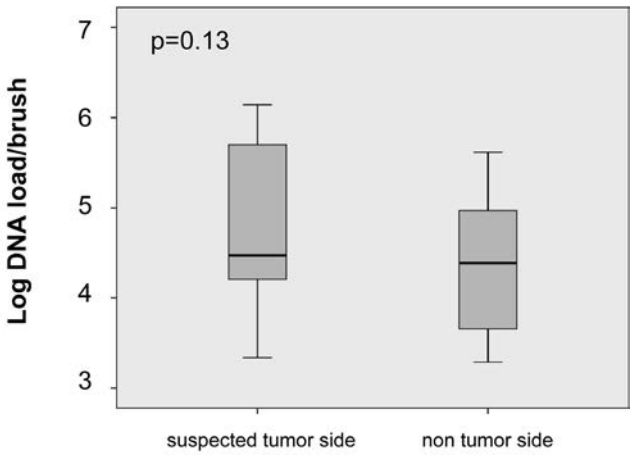


Figure 3 | Viral DNA load of bilateral side nasopharyngeal brushing.
Bilateral brushing (n=20) was performed at the site of suspected tumor location and the non-tumor site. The mean viral DNA load was $3.4x10^5$ vs $7.1x10^4$ copies per brush, respectively (p=0.13), which indicates that single brushing at the site of enlarged neck node may be more representative for detecting NPC presence.

Comparison of viral DNA load at diagnosis and two months post treatment

In 69 patients the effect of the therapy on the viral load was analyzed by comparing the viral DNA load in NP brush and whole blood at diagnosis and 2 months post-treatment. The median EBV-DNA load in NP-brushing at diagnosis was $9x10^5$ copies/NP brush and decreased after two months post-treatment to a median of $3x10^3$ copies/NP brush indicating a 300-fold reduction (Figure 4). Initially 96% of patients had a viral load > COV level in the NP brush, but after treatment this dramatically reduced to 39.4%. Similarly, the level of EBV-DNA in whole blood was significantly lower post-treatment with a reduction of 27-fold ($p<0.001$ for both), and the percentage of patients with a viral load >COV in the circulation dropped from 51% to 8.8%. Although the fold reduction in viral DNA load in both NP brush and blood samples reflected the treatment response, irrespective of the regimen used (see below), the level of EBV DNA at diagnosis did not have any predictive value for treatment outcome.

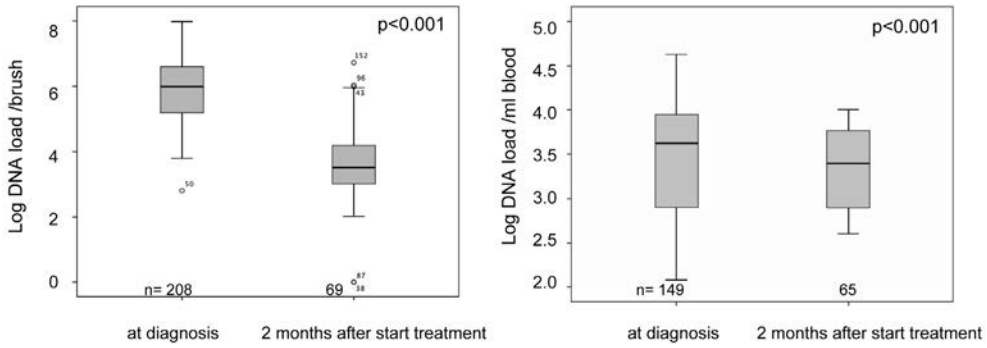


Figure 4 | Viral DNA load in NP brush and whole blood at diagnosis and after treatment.
A. EBV-DNA load in NP-brushings (copies/brush). **B:** EBV-DNA load in whole blood. There was a significant decrease in EBV-DNA load in both NP-brushings and whole blood at diagnosis compared to 2 months post-treatment for samples paired before and after treatment ($p<0.001$).

Patients treated with neoadjuvant plus hyperfractionated radiotherapy had a median value of $8.9x10^6$ copies/brush at diagnosis which decreased after therapy to $1.7x10^5$ copies/brush ($p=0.006$). Patients treated with concurrent chemoradiation had a median DNA viral load in brushings of $1.9x10^7$copies/brush at diagnosis decreasing to $5.8x10^4$copies/brush after therapy ($p=0.049$). Only one patient with neoadjuvant and radiotherapy had increased DNA viral load brush post-treatment ($9.5x10^5$ copies/brush) and one patient had increased viral load in whole blood at 2 months post-treatment, both linked to progressive disease.

Based on response to treatment 41 patients had a complete response at 2 months post-treatment as judged by clinical examination plus a negative CT-scan and negative biopsy. These patients had a post-treatment median viral DNA load in the NP brushing of $3.0 x10^3$ copies/brush, a significant difference compared to the pre-treatment value of $1.7 x10^6$ copies/brush in this group ($p=0.013$) (Figure 5). In 22 patients with partial response the median EBV-DNA load in NP brush pre-treatment was $1.3x10^6$ and post treatment $3.2 x10^3$ copies/brush ($p = 0.14$). For whole blood samples most cases with an initial positive DNA load, the EBV DNA load became undetectable after 2 months after start treatment for both complete and partial responses. Two of three patients with progressive disease post-treatment showed a median of EBV-DNA load in brush being below COV, whereas the post-treatment median level in blood was above COV in all 3. Two patients died within 2 months post-treatment and their DNA viral load brush was above COV, whereas the viral load in the whole blood was negative. No significant difference was observed for EBV-IgA serology levels at diagnosis compared to two month post-treatment, neither for VCA-p18 nor for EBNA1 antibody levels individually, nor for different treatment regimens (data not shown).

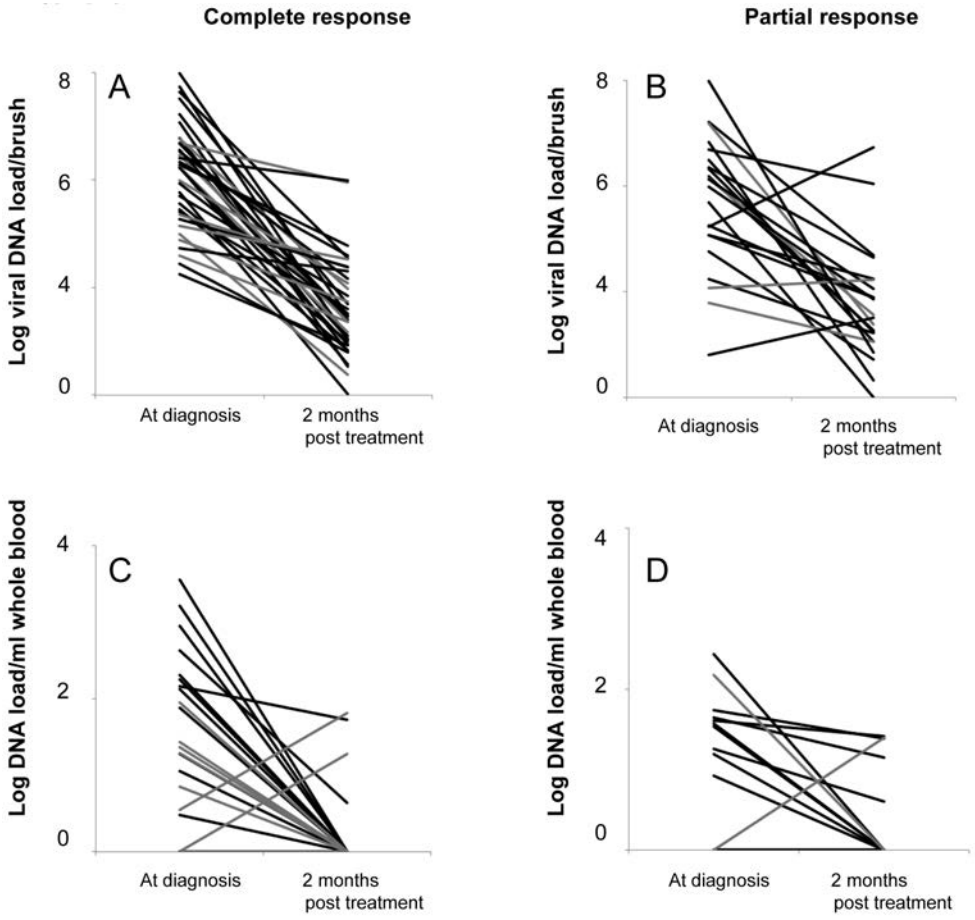


Figure 5 | Viral DNA load of NPC patient samples before and 2 months post treatment in relation with treatment response.

NPC patients treated with concurrent therapy are given in black and patients receiving neo-adjuvant therapy are presented in gray. Presented is EBV DNA load in **A:** in brushes of NPC patients with complete response, **B:** in brushes of NPC patients with partial response, **C:** in whole blood of NPC patients with complete response, **D:** in whole blood of NPC patients with partial response. Complete response is defined as complete disappearance of locoregional disease by physical examination or X-Ray and CT Scan and endoscopic examination and a negative biopsy at 2 months post treatment; partial response is defined by reduction of disease by 30% or more based on clinical examination or X-Ray and CT-Scan. If the disease shows a slight increase in size or extends after treatment it is defined as progressive disease.

DISCUSSION

Pathological examination for diagnosis of NPC requires an invasive biopsy that is painful and cannot be repeated easily. A less invasive diagnostic procedure by using NP-brush sampling would be preferred, also for assessment of post-treatment tumor activity. This NP-brush procedure may also be combined with detection of aberrant EBV-IgA serology in screening approaches of patients at (family) risk or having symptoms suggestive of early-stage NPC[25]. In this study we evaluated minimal-invasive NP-brushing with quantification of EBV-DNA load for primary NPC diagnosis and assessment of treatment response relative to the standard biopsy taken in parallel[14]. We also measured EBV-DNA load in whole blood and VCA-p18 and EBNA1 specific EBV-IgA serology in simultaneous venous blood samples[21]. We demonstrated that measuring EBV DNA load in NP brushings provides a highly specific tool for primary NPC diagnosis with minimal patient discomfort, giving better sensitivity/specificity compared to EBV-IgA serology and EBV-DNA load in blood, as detailed in Table 4. Because most patients in this study presented with advanced stage NPC, the utility of NP brushing for detecting early stage NPC remains to be defined. In ongoing studies in patients with persistent head and neck complaints, non-responsive to antibiotic or anti-allergy therapy, we are currently validating this method for identification of early stage NPC. The diagnostic utility of NP brush may be further increased by assessing a combination of molecular carcinoma markers in the same brush material, including tumor-specific EBV-RNA transcripts[14], host genomic methylation patterns[15,16] and other genetic abnormalities linked to NPC.

Although detection of NPC at early stage is important for the patient outcome, diagnosis is often difficult because of the non-specific nature of the clinical symptoms and difficulty in visualizing the nasopharynx[4]. Only 12.2% of our patients presented with early TI-IIa stage, whereas 24.6% presented with TIIb with tumor already invading into the parapharyngeal area giving worse prognosis compared with localized disease limited to the nasopharynx. The majority (89%) of patients, however, already had parallel enlargement of the regional lymph node indicative of advanced (late) stage (Table 1), which is typical for most endemic regions[3]. This situation reflects the need for novel diagnostic procedures for regular testing of NPC risk populations, such as family members of NPC patients and patients with chronic head and neck complaints suggestive for early stage NPC[25,26].

NP brushings from NPC patients frequently contain extremely high levels of EBV-DNA compared to other clinical conditions, including EBV-related non-NPC head & neck cancers (Figure 1), confirming previous studies[28,29]. Over 95% of our NPC patients had a brush containing viral loads above COV. A negative result (5%) of DNA EBV viral load brush might be caused by absence of cancer cells or obscured by blood, by tumor detritus or due to improper sampling. Both primary and recurrent cancers may be located deep under the

overlying mucosa and early lesions not invading the nasopharyngeal surface can be difficult to detect when biopsy or brushing is done too superficially[30]. Contrary, NPC tumor-derived EBV-DNA from submucosal locations may reach the surface (shedding) leading to detectable aberrant levels in the brush. Our data indicate that NP brushing combined with quantitative real-time PCR directly reflects carcinoma-specific EBV involvement at the anatomical site of tumor development. The NP brush may greatly reduce the number of invasive NP biopsies required when applied for diagnosis and follow-up monitoring. Since bilateral brushing might be necessary for EBV tumor detection Tune et al. originally recommend bilateral brushing as a routine to avoid missing small, localized tumors[13]. We performed brush and biopsy sampling under endoscopic guidance for all patients, which may be a preferred procedure for accuracy of sampling. Our data on bilateral brushing (Figure 3) indicate that random brushing of the nasopharyngeal cavity may be adequate, supporting the general applicability of the brush technique for NPC diagnosis, without the need for endoscope guidance. Blind brushing may be done in the NP area on the side of the neck node at the lateral pharyngeal recess, because this is the most common site for early disease. However this needs to be further evaluated.

The level of discomfort and pain was analyzed between brushing and biopsy procedure in 57 suspected NPC patients,. The NP brushing procedure was well tolerated and none of the patients or controls complained of negative effects like pain or bleeding etc. In contrast, the biopsy procedure frequently associated with excessive bleeding and pain. In 11 patients repeated biopsies were needed to pathologically verify the presence of tumor cells, whereas EBV-DNA load in the parallel brush was above COV at the first sample with 2 patients having very high viral loads. Overall, NP brushing proved to be a specific and minimal invasive diagnostic tool for NPC diagnosis. However the possibility remains that a deeply located tumor is missed by the NP bushing procedure, while a deep biopsy may be able to yield sufficient number of tumor cells for making a diagnosis[30]. This can only be confirmed in more extensive studies.

The sensitivity, specificity, PPV and NPV for detecting EBV-DNA load above the pre-determined clinical cut-off level in whole blood (WB), being 71%, 50%, 86% and 20%, respectively (Table 2), were low compared to the NP brush values. This confirms a previous independent study showing that many patients have only minimal (50%) or even negative (25%) EBV DNA levels in blood[21]. Circulating EBV-DNA does not reflect intact circulating tumor cells, because EBV-RNA transcripts from either BART, LMP2 or BARF1 reading frames were not detectable in the whole blood samples. EBV-DNA in blood reflects apoptotic release of DNA fragments with an average size of 150 bp or less, which are rapidly cleared from the circulation[18,28,29]. High EBV-DNA blood levels therefore may reflect on going tumor apoptosis

and necrosis rather than a growing tumor mass[21]. Our quantitative data on circulating EBV-DNA load differ from the initial studies by Lo et al. in Hong Kong[17,18], as detailed elsewhere[22]. Pre-treatment level of circulating EBV-DNA is considered to be a prognostic factor for NPC[19,28,29,31]. Others showed that circulating EBV-DNA levels may correlate with stage of disease[18], which was not observed in this study. However, percentage of NPC patients with elevated EBV-DNA levels in blood or plasma differ between studies and procedures are not well standardized. In this study elevated EBV DNA load in blood above the clinical COV of 2000 copies/ml was detected in only 50% of the NPC patients. Some patients with extensive clinical disease (Stage IVB) completely lacked circulating EBV DNA, despite having high EBV-DNA levels in the NP-brush collected at the same time. These observations confirm prior findings that EBV-DNA load in blood may not provide strong diagnostic information[21]. Tong et al found that T1 tumors had a significantly lower EBV DNA level as compared to cases with locally more advanced disease[32]. In this study only a tendency of increasing DNA viral load between early and advanced tumor stage was observed (Figure 2). In addition, we found no correlation between the level of EBV-DNA in blood or NP brushing at diagnosis and the clinical response at 2 months post-treatment. Therefore the initial EBV-DNA load values may not be taken as a prognostic marker.

At two months after treatment the level of EBV-DNA load in brush and whole blood showed a significant decrease in most cases, being clinically relevant and reflecting reduced tumor activity. For viral DNA load in NP brushings there is a substantial reduction (43-fold), similar to whole blood (27-fold reduction). We did not find any correlation between type of treatment ,treatment response and the fold reduction of viral DNA load. Two patients died before treatment was finished both having an initial high EBV-DNA load in whole blood and distant metastasis pointing to an initial poor prognosis. Post-treatment EBV-DNA levels have proven to be a strong predictor for relapse and survival in larger studies[17,19,31,33-35]. The time point of 2 months follow-up chosen for this study may be too short to permit complete disappearance of treatment induced tumor-related EBV activity in complete responders. More long-term follow-up is needed to define the clinical relevance of persisting EBV-DNA levels in NP brush samples.

In summary, this study demonstrates that EBV-DNA quantification in NP brushings is a promising approach for NPC diagnosis and post-treatment monitoring and may reduce the number of invasive NP biopsies required. Although pathological examination for definite NPC diagnosis remains needed, molecular testing of NP brush material provides a promising and minimally invasive alternative requiring further validation. NP brush sampling is suitable for follow-up monitoring to measure EBV-DNA load dynamics during and after treatment aiming at detection of progressive or recurrent disease without significant discomfort for the patient.

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Chapter 5

Epstein-Barr virus markers in relation to treatment and clinical response in nasopharyngeal carcinoma patients

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ABSTRACT

Aim of study: Nasopharyngeal carcinoma (NPC) is causally related to Epstein-Barr virus (EBV). A prospective study was performed to analyse the implementation of non-invasive EBV DNA load in nasopharyngeal (NP) brushings, whole blood (WB), and EBV-IgA serology for follow-up monitoring of NPC patients under standard chemoradiation treatment in Jakarta, Indonesia. The diagnostic methodology was evaluated for prediction of recurrent disease in the head and neck region and for the dynamics of viral biomarkers in NP brushes and WB from patients with NPC during and post treatment.

Methods: Sixty-eight NPC patients were treated with standard chemoradiotherapy. Patients were sampled by NP brush and blood collection. EBV DNA load from NP brush and WB was measured by quantitative PCR. In addition, IgA response against VCA p18 and EBNA1 were determined by ELISA.

Results: We evaluated 68 patients at 8 week post treatment, 2 years post treatment and before relapse. WHO-III was the most common histopathology found (87%). Bilateral node (N2) was found in 22% of cases while 34% (23 cases) had affected lymph nodes with diameter >6 cm. Six cases with supraclavicular lymph node involvement had poor prognosis. Sixty-seven cases were treated by neoadjuvant chemotherapy followed by radiotherapy. Only 40 patients could be used for analysis of all 4 markers at diagnosis till 8 weeks post-treatment. At later times during follow-up only 30 patients were evaluated for viral DNA load in brush, 20 patients for WB viral DNA load, and 29 patients for both IgA VCA P18/EBNA1 due to incomplete sampling. Strong decreases in EBV DNA load in brush reflected good response to therapy. In initially positive cases WB EBV DNA load had significant correlation with treatment response, at least 2 years post-treatment. In patients with both partial response and progressive disease EBV DNA load in brush increased prior to development of distant metastasis (DM). Both EBNA1 and VCA-P18 IgA levels increased when DM developed, however not significantly. In most cases increase of EBV DNA load in brush was not followed by increased EBV DNA load in blood when DM developed. In our study IgA-EBNA1 was better predictor of metastasis while IgA-VCA was more stable. At diagnosis serology did not predict for survival.

The limited numbers of uniformly treated subjects and irregular sampling precluded overall accurate monitoring of NPC treatment outcome. Further study is required.

Conclusion

Although viral DNA dynamics correlated well with clinical performance during and shortly after treatment, neither the level of viral DNA load in brush or blood nor IgA VCA-P18 and EBNA1 serology reliably predicted recurrent disease.

INTRODUCTION

Nasopharyngeal Carcinoma (NPC) is the most prevalent head and neck cancer in Indonesia with an estimated 12,000 new cases each year[1]. NPC is a highly prevalent malignant disease and a leading cause of death in several regions of Southeast Asia, Northern Africa and in native populations of the Arctic region. However in most parts of the world NPC is a rare malignancy. Although NPC is a radiosensitive tumor, long-term survival of patients with advanced disease remains poor. Concurrent chemoradiotherapy (CCRT) showed to improve outcome for stage III–IV nasopharyngeal carcinoma (NPC) patients compared with radiotherapy (RT) alone. For stage II adding chemotherapy gives 5 years overall survival of 94.5% for CRT vs 85.8% for RT alone[2]. However, in Indonesia where 99% of the patients present at late stage[1], curative treatment options are limited. Data from the intergroup 0099 study showed that CRT in patients with advanced disease resulted in a 5 years progression-free survival and overall survival around 83% and 68%, respectively[3]. The results in Indonesia of a recent study from Yogyakarta revealed a dramatic lower disease outcome. Chemoradiation therapy resulted in a median of 21 month overall survival and the 3 year survival was below 30%[4], which is similar to the results in Jakarta showing a 3 years survival of 53% and a 5 years survival of 38% (Soehartati, personal communication). Treatment failure in patients with advanced disease at intake is often due to a high rate of local recurrence and distant metastasis, certainly in areas with poor treatment compliance and limited facilities.

Undifferentiated NPC is associated with Epstein-Barr virus (EBV) in almost 100% of the cases. The EBV genome is present in a clonal state in all tumor cells from primary and metastatic NPC indicating that EBV is an early event in the carcinogenesis[5,6]. Therefore EBV presence and activity might be used as a diagnostic marker and indicator of disease activity and treatment response. The quantification of EBV DNA load in plasma of patients with NPC by quantitative real-time PCR has been shown as a usefull marker in the diagnosis of NPC[7,8]. Subsequently assessment of viral load in the circulation has been shown to be valuable for prognosis and monitoring during and post treatment of NPC patients[8–12]. In addition, clearance rate of EBV DNA from the plasma could be used as early predictive marker in risk-assessment for metastatic or recurrent disease[13]. In patients with NPC, circulating EBV DNA in plasma is considered to originate from apoptotic tumor cell fragments and not from circulating NPC tumor cells[14]. Unfortunately, lack of standardization leads to considerable variation in plasma DNA levels between different laboratories[15]. Although plasma EBV DNA values may have value for NPC diagnosis and treatment monitoring, measuring EBV DNA load in whole blood, which is a preferred method in EBV driven lymphoid malignancies[16] has revealed less promise in NPC[17]. Further studies are needed to define the role of circulating EBV DNA

(fragments) in NPC diagnosis and prognosis. Local tumor behaviour in the nasopharyngeal (NP) space can be adressed by analysis of EBV markers such as genomic DNA load or RNA expression profiling in non-invase NP brushings[18–21]. NP brushing revealed high viral load in NPC cases at diagnosis compared to non-NPC and regional healthy controls[18] and can be used to visualize NP tumor cells in brush-based cytology[22]. Therapy response can be assessed by monitoring viral load dynamics in NP brushings, since 2 months post treatment EBV DNA levels dropped dramatically, which was paralleled by a steep decrease of EBV DNA load in blood[23]. The low persistent viral load after treatment could give the opportunity of detecting the development of recurrent disease by increasing viral load in the brush. Serology is an excellent marker for NPC diagnosis and may be used as well for follow-up monitoring of patients post treatment[24,25]. Two months after the end of treatment the IgA response of NPC patients is commonly sustained at high levels and a broad spectrum of serum EBV antibodies can be observed[25–29]. Further follow-up post treatment might indicate whether the antibody levels decrease to levels observed in healthy individuals as suggested by a previous case study[24] and whether they provide a good prediction for tumor relapse[25].

In this study, a long term follow-up of patients with EBV positive NPC post treatment was performed. Viral biomarkers were assessed during and post treatment in relation to monitoring treatment responses and development of recurrent disease. EBV DNA load was assessed in NP brushings as well in the circulation. Serology was analyzed in the plasma by measuring the level of IgA against VCA-p18 and EBNA1 antigens. These viral markers were analyzed at time of diagnosis, 2 months and 2 years post-treatment and were related to clinical outcome at last contact.

METHODS

Patients

Between 2006 and 2009 228 patients presenting with symptoms suspected for NPC at the ENT clinic of Dr. Cipto Mangunkusumo Hospital, Universitas Indonesia (Jakarta, Indonesia) were enrolled in this study. From the 228 patients with confirmed NPC, 68 patients could be included in this study with a follow-up of more than 2 months post treatment. After initial treatment many patients dido not return to the clinic, which explains the high loss of follow up. The inclusion criteria were confirmed NPC by histopathological examination of the biopsy from the suspected site of nasopharynx, and presence of EBV as indicated by EBER-RISH using a commercial PNA-based hybridisation kit (Dakocytomation, Glostrup, Denmark). This study was approved by the ethics committee of the Faculty of Medicine University of Indonesia, Jakarta. Written informed consent was obtained from all patients.

The tumor stage of the patients was performed based on TNM staging according to the 2002 Union Internationale Centre le Cancer (UICC)/ the American Joint Committee on Cancer staging criteria[30,31]. Patient characteristics are summarized in table 1.

Table 1 | Patients characteristics at diagnosis (n=68). Neo: neoadjuvant, CT: chemotherapy, RT: radiotherapy, HPR: hyperfractionated, Conc CRT: concurrent chemoradiotherapy.

		n	%
Sex	Male	43	63
	Female	25	37
Histopathology	WHO I	8	12
	WHO II	1	1
	WHO III	59	87
Age	<10	0	0
	10-20	7	10
	21-40	26	38
	>40	35	51
T Stage	T1	7	10
	T2a	6	9
	T2b	19	28
	T3	17	25
	T4	19	28
N stage	N0	7	10
	N1	17	25
	N2	15	22
	N3a	23	34
	N3b	6	9
N Stage	N0	6	9
	N+	62	91
M Stage	M0	67	99
	M1	1	1
Stage AJCC-UICC	1	1	1
	2b	6	9
	3	18	26
	4a	13	19
	4b	29	43
	4c	1	1
Stage summarize	Early	1	1
	Advance	67	99
Type treatment	Neo CT+RT	17	25
	Neo CT+HPR	30	44
	Neo CT+Conc CRT	20	29
	Chemo	1	1

Treatment and follow up

All patients were treated with radiotherapy on the primary tumor and the neck region. The total dose administered was 66 to 70 Gy, given in 6 to 8 weeks, by conventional fractionated or partially hyperfractionated accelerated radiotherapy. All patients were treated with additional chemotherapy (Table 1). Seventeen patients were treated with neoadjuvant chemotherapy followed by conventional radiotherapy, 30 patients were treated with neoadjuvant followed by hyperfractionated radiotherapy and 30 patients received neoadjuvant followed by concurrent chemoradiation, and one patient was treated by full dose chemotherapy only. Neoadjuvant/adjuvant chemotherapy consisted of 5-FU (1000 mg/m^2 day 1-5) and cisplatin (100 mg/m^2 in 3 courses every 3 weeks). Concurrent chemotherapy consisted of cisplatin (40 mg/m^2), and was weekly administered during radiotherapy[32,33]. Tumor response was evaluated at 8 weeks post-treatment according to the WHO criteria and NCCN protocol. Clinical evaluation of patients was done every 3 months and consisted of anamnesis, physical examination and fiber nasoendoscopy. Patients, who developed symptoms or signs suspicious for local recurrence or metastasis, were investigated further with nasopharyngeal biopsy and imaging. Complete response was defined as complete disappearance of all lesions, confirmed by physical examination, including endoscopy of the nasopharynx, CT scan of the head and neck and X-ray examination every year or if needed. A reduction of the tumor by 30% or more was defined as partial response, and progressive disease (PD) was defined as a tumor volume increased in size of 20% or the appearance of any new lesion (according to RESIST 1.1 criteria)[34–36]. Survival was defined as the time from diagnosis to the date of death or censored at the date of last report when the patient was still alive. Patients were requested for nasopharyngeal brush sampling and peripheral blood sampling every week during treatment and every 3 months after completion of treatment.

Nasopharyngeal brushings sampling

NP brushing was performed under rigid or flexible nasoendoscopic guidance by experienced ENT specialists and ENT resident trainees. Endoscope-guided NP brushings were taken under local anaesthesia (1% Lidocaine spray, Astra Zeneca, Waltham, USA). Fiber nasoendoscope was used to evaluate the entire nasopharynx and photograph images were taken routinely from the site of tumor involvement. Other procedures are the same as described in a recent parallel study[23]. NP Brushes were mixed well in 4 ml NucliSens lysis buffer (LB) (BioMerieux) and aliquots of 1 ml were stored at -80°C.

Peripheral blood sampling

Five ml peripheral blood was collected. From the whole blood 100 µl was added to 900 µl LB. The remaining blood was used for preparing plasma by centrifugation for 5 min. Plasma samples were stored at -20°C until use.

Quantitative EBV DNA load PCR

DNA was isolated from NP brushings and whole blood samples in LB by silica-based nucleic acid extraction as described previously in detail[17,18,37,38]. Reagents for the DNA isolation procedure were purchased from BioMerieux and EBV DNA loads were determined by quantitative real-time PCR (LightCycler 480, Roche) targeting a well conserved 99-bp region of the BKRF1 gene encoding Epstein-Barr nuclear antigen 1 (EBNA1). The clinical cut-off value (COV) for viral load was 2,300 EBV DNA copies per brush and the clinically relevant COV of the PCR for blood samples was 2,000 EBV DNA copies/ml, which was based on low EBV DNA loads normally detected in the blood of healthy EBV-seropositive donors as determined in previous studies[16–18]. The quality of isolated human genomic DNA from NP brushing specimens (sampling control) was confirmed by quantitative real time PCR targeting a 197 bp fragment of the human ß-globin gene[17,18]. Levels above COV were considered positive for statistical analysis.

VCA-p18 and EBNA1 IgA ELISA

Immunoglobulin-A (IgA) antibody levels against EBV in serum of patients with NPC were measured by ELISA. Defined immunodominant epitopes of EBNA1 and VCA-p18 in form of synthetic peptides were used in well-standardized ELISA assays as described previously[39]. All samples were tested in duplicate next to a panel of sera from healthy individuals. Sample values were normalized by dividing the mean OD_{450} value of duplicates by the mean + 2x standard deviation of a standard set of sera from healthy individuals (n=4). The clinical cut-off value (COV) was 1 and samples and values of >1 were considered as positive.

Statistical analysis

The decrease of EBV DNA copies during treatment in relation to treatment outcome was analyzed by one-way ANOVA. The relation of clinical outcome (disease free or progressive disease/death by disease) to the viral biomarkers arranged by the COV value was performed by a Pearson Chi square assay which was when the factor was above 3.84. The survival was analyzed by Kaplan Meier curves and differences were calculated by Mantel-Cox analysis. All statistical analyses were performed using SPSS software. A p-value below 0.05 was considered to be significant.

RESULTS

Demography data of patients

Sixty-eight patients with confirmed NPC could be included in this study with a follow-up of more than 2 months post treatment. Patient characteristics are presented in table 1. The male female ration was 43:25 (2:1), similar as reported by others before[40]. Mean age of patients was 40 years, with a major group above the age of 40 years (51%), and 38% between the age of 21 and 40 years and 21% being below 21 years of age at presentation to the clinic. Only 1 case presented at early stage of disease, while the other 67 patients had already advanced stage of disease at intake. Primary tumors were at T3 and T4 stage, in 17 (25%) and 19 (28%) of patients, respectively. Twenty three patients entered with an enlargement of the neck of more than 6 cm bilateral and 6 (9%) patients already had a supraclavicular node. The treatment consisted of neoadjuvant followed by chemoradiation hyperfractionation treatment for 30 patients (44%) with total doses of 70 Gy. Seventeen patients (25%) were treated by neoadjuvant followed by radiation. Later-on treatment was switched to concurrent chemoradiation in 31 patients (29%). One patient received palliative chemotherapy only.

At 2 months post-treatment 40 patients (59%) could be defined as having a complete response, while after 2 years follow-up only 24 patients (35%) were disease free (Table 2). At last contact by phone 26 patients (38%) were determined as disease free. Three patients died from the disease within 2 months post-treatment and at 2 years post-treatment 10 patients were lost by death caused by NPC. At last contact by phone the total number of disease related deaths increased up to 14 patients (Table 2).

Table 2 | Therapy responses at 2 months, 2 years after therapy and at last contact by phone. Many patients could not be evaluated at the 2 years after start treatment and were phoned at the end of this study for disease status (till 10 years after diagnosis).

	2 months after treatment			2 years after treatment		Last contact	
	n	(%)		n	(%)	n	(%)
Complete response	40	59	Disease free	24	35	26	38
Partial response[1]	22	32					
Progressive disease	2	3	Progressive disease	15	22	28	41
Death	3	4	Death	10	15	14	21
LTFU	1	1	LTFU	19	28	0	0

1: not analyzed after 2 years

LTFU: Lost to follow up

Analysis of EBV marker levels in NPC patients at diagnosis till 2 years post-treatment.

Patients were sampled at diagnosis, during treatment and up till 2 years post treatment. Samples included nasopharyngeal (NP) brushings with tumor cells from the nasopharyngeal area, and whole blood to determine the viral DNA load and plasma for analysis of the IgA antibody response against viral latent (i.e. EBNA1) and lytic (i.e. VCA-p18) antigens. We evaluated these 4 viral markers in relation to diagnosis, treatment responses and for prediction of recurrent disease. The viral load in NP brushes showed high levels of EBV DNA (median 1.0 $x10^6$ copies DNA EBV/NP brush) at diagnosis, which decreased significantly ($p=0.002$) at 2 months post-treatment (median $3.3x10^3$ copies DNA EBV/NP brush) (Fig. 1A). This decrease in viral load of >300 fold post-treatment was similar to a previous analysis in the whole patient group (n=268)[23]. However, during further follow-up the viral load in most brushes remained above the COV value, albeit at relatively low values. At 2 years post-treatment viral DNA load in the majority of samples was below COV (median $1.9x10^3$). The viral load levels in the NP brush were not indicative for relapse, since most patients did not develop a viral load above COV when developing relapsing disease, although the overall population showed a minor shift towards higher viral load (median $7.7x10^3$ copies DNA EBV/NP brush) (Fig.1A). Most relapses concerned a distant metastasis, which may not be detected by a local NP brushing. Four patients showed a local relapse of which 3 patients had a viral load above the COV ($7.1x10^3$, $7.7x10^3$ and $3.0x10^6$, respectively).

At diagnosis EBV DNA load in whole blood of patients with NPC had a median value slightly below the COV of 2000 copies per ml blood (median $1.7x10^3$ copies/ml) (Fig.1B). Patients with detectable viral load in the circulation at diagnosis showed a decrease in viral load to low or undetectable levels at 2 months post-treatment. Two years post treatment viral load remained negative in most samples as well as in patients just before relapse. Since many samples had negative results, the quality of the DNA was confirmed by a quantitative ß-globin PCR. All samples revealed DNA levels exceeding 10^6 cells confirming proper DNA isolation and amplification procedure, and absence of sample-related PCR inhibition.

The antibody responses against EBV were analyzed as IgA antibodies to the lytic VCA-p18 antigen and the viral latency protein EBNA1. At diagnosis normalized levels of IgA antibodies against VCA-p18 and EBNA1 were higher than the COV of 1 (median respectively, 2.0 and 3.1) (Fig.1C). After 2 months and up till 2 years post treatment both IgA levels remained elevated. Antibody responses against VCA-p18 and EBNA1 were not indicative for tumor relapse, since they showed a similar and stable level in samples just before recurrence (Fig.1D)

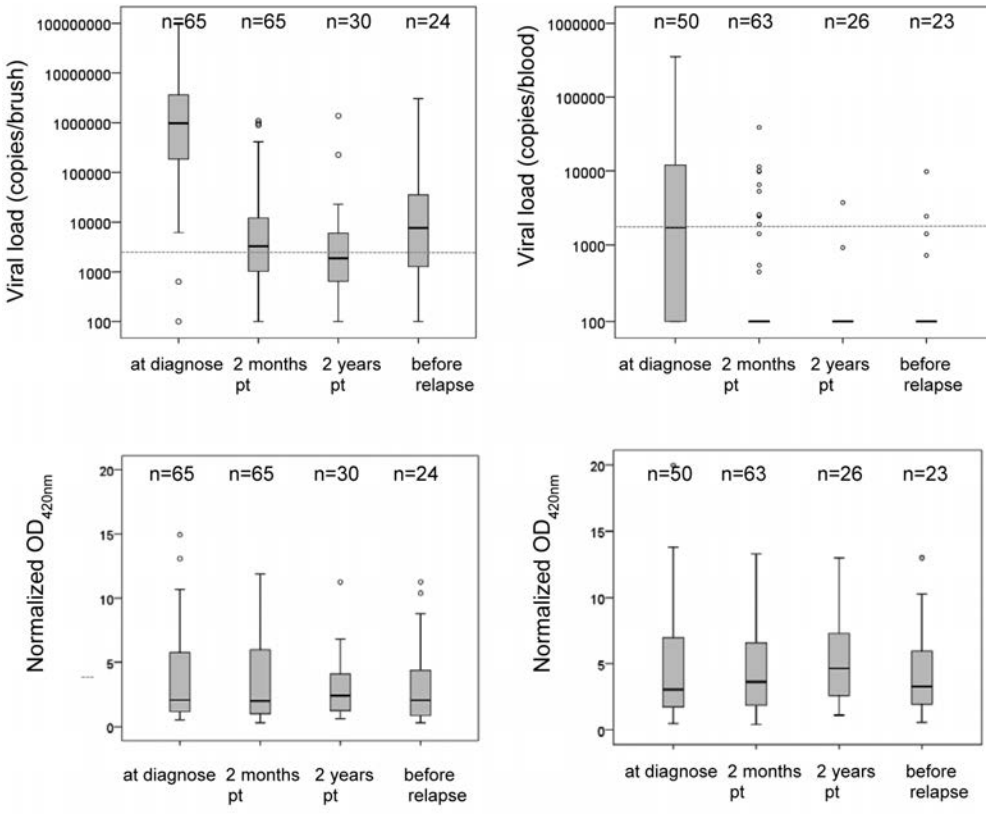

Figure 1 | Level of 4 biomarkers at diagnosis, 2 months and 2 years post treatment (pt), and at sample before or at recurrence. A: Viral load was high at diagnosis and decreased post treatment (p=0.002), at recurrence viral load was slightly increased, B: Viral load in the circulation was higher at diagnosis and was mostly undetectable after therapy (p=0.013). C: IgA responses against VCA-p18 (p=0.598) and D: EBNA1 remained similar before and post treatment in the total group of patients (p=0.495). The dashed lines indicate the COV for each biomarker: COV for viral load in brush 2,300 EBV copies per brush, in circulation 2,000 EBV copies per ml whole blood, for serology the COV is 1.0.

Relation of clinical outcome and biomarkers at defined time points

The COV values of viral biomarkers in this study were previously defined for discriminating patients with NPC at diagnosis from patients with other head and neck diseases and regional healthy controls[17,18,23]. In this study the use of COV values was studied for the prediction of clinical outcome as defined at last contact (Table 3). For statistical analysis patients were divided in a group without disease at last contact and a group of patients with progressive disease combined with patients who died from NPC. A relation between clinical outcome

with a biomarker was analysed by the Pearson Chi-square test. At diagnosis 62 out of the 64 patients (97%) had a viral load above COV in the brush, which confirms the value of this marker for diagnosis[18]. However the viral load in brush above COV was not useful for the prediction of overall clinical outcome. This was observed as well for viral load in blood, although not all samples showed detectable EBV DNA in the blood at diagnosis. IgA antibody responses against VCA-p18 and EBNA1 also were suitable for diagnosis being mostly above COV, but did not relate with clinical outcome. At 2 month post-treatment 22% of patients had a brush viral load in brush below COV and were disease-free. However 26% of patients with brush viral load below COV had progressive disease. In 10 patients (15%) who died from disease the viral load in brush was above COV, indicating progressive disease.

Table 3 | Analysis of 4 viral markers from patients with NPC 2 years posttreatment in relation to treatment response. Viral load was determined in NP brushes and in whole blood. Serological markers were the IgA antibody responses against VCA p18 and EBNA1 as determined by ELISA. COV values for viral load were 2,300 copies per brush, 2,000 copies per ml blood and for serology COV was 1. The relation of the viral biomarkers with clinical outcome was considered to be significant ($p<0.05$) as the factor was above 3.84

	Viral load in brush				**Viral load in whole blood**				**Antibody level IgA VCA-p18**				**Antibody level IgA EBNA1**			
	below COV		**above COV**		**below COV**		**above COV**		**below COV**		**above COV**		**below COV**		**above COV**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
At diagnosis	n=64				n=47				n=57				n=60			
Complete response	1	2	24	38	12	26	7	15	4	7	20	35	4	7	21	35
Progressive disease	0	0	27	42	6	13	11	23	3	5	18	32	2	3	21	35
Death	1	2	11	17	6	13	5	11	4	7	8	14	2	3	10	17
Pearson chi square	0.10				1.87				0.18				0.26			
2 months after therapy	n=68				n=64				n=66				n=59			
Disease free	15	22	11	16	23	36	2	3	3	5	23	35	3	5	23	39
Progressive disease	18	26	9	13	21	33	5	8	6	9	22	33	4	7	24	41
Death	4	6	10	15	10	16	2	3	6	9	6	9	1	2	11	19
Pearson chi square	0.10				1.34				0.93				0.01			
2 years after therapy	n=30				n=26				n=30				n=30			
Disease free	9	30	10	33	16	62	1	4	1	3	18	60	0	0	19	63
Progressive disease	9	30	1	3	8	31	0	0	2	7	8	27	0	0	10	33
Death	0	0	1	3	1	4	0	0	0	0	1	3	0	0	1	3
Pearson chi square	3.44				0.55				1.29				na			

For viral load in whole blood 36% of patients that were disease-free post-treatment showed levels below COV. However, viral load was below COV in 21 (33%) patients with progressive disease and in 10 patients (16%) who died. Serology showed no trend in predicting clinical outcome, since most samples at 2 months post-treatment for IgA VCA-p18 and EBNA1 assays, were above COV levels independently of the disease state and remained rather stable at this level till 2 years post-treatment (Table 3). At 2 year post-treatment all 4 biomarkers showed no relation with patient clinical outcome. Therefore we conclude that at fixed time points post-treatment an analysis of EBV markers by COV values was not useful in predicting clinical outcome. The dynamics (change in level over time) of the viral biomarkers could add to the understanding of tumor behavior.

Dynamics of 4 viral biomarkers

In 30 patients the dynamic changes in the level of the 4 viral markers over time was evaluated per individual patient starting at diagnosis untill 2 years post-treatment (Fig. 2). The dynamics did not reveal a similar pattern in all patients. Therefore 3 patients were chosen as typical examples representing patients with complete response, partial response and progressive disease after treatment (illustrative examples are shown in Fig. 2). In the patient with a complete response (Fig. 2A) viral load in the brush was extremely high at diagnosis reaching 43 million copies EBV-DNA per brush and had a tremendous (more than 10,000 fold) decrease in viral load at 2 months post treatment. After treatment the viral DNA load reached a stable level below the COV with a little increase at a single point at 95 weeks post-diagnosis. In the circulation viral DNA load was negative at diagnosis and remained undetectable in follow-up. IgA antibody responses against EBNA1 were very high at diagnosis and even reached plateau levels in the assay, which rapidly declined after the end of treatment. The VCA-p18 levels were lower and showed a gradual decrease during follow-up.

The patient with partial response had a 135,000 fold decrease in viral DNA load in the brush treatment declining to levels below COV even before the end of treatment (Fig. 2B). Unfortunately the patient developed a distant metastasis, which was preceded by a small increase in viral DNA load in the brush which again decreased during subsequent treatment. The viral DNA load in the circulation of this patient was undetectable at all times during the whole analysis. The levels of IgA antibodies against both VCA-p18 as EBNA1 remained stable. Only EBNA1 was highly elevated, whereas VCA-p18 antibody levels were around the COV. Treatment as well as the development of a distant metastasis did not influence antibody levels.

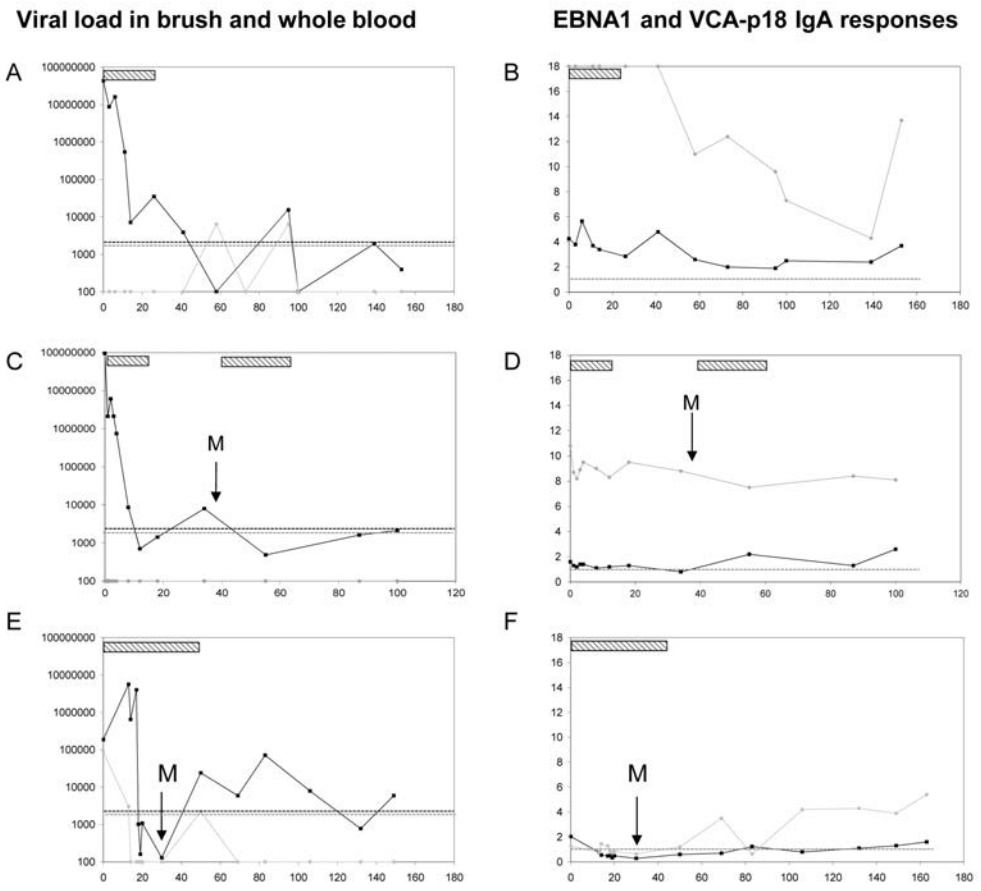

Figure 2 | Dynamics of EBV DNA and serology markers at start, during and post treatment are presented. Typical examples are shown for patients having complete response (A, B) and partical response (C, D) and progressive disease (E-F) as measured after 2 months post treatment. The dashed bar indicates the period of treatment. The amount of EBV genomes detected in NP brushes (black line) decreased significantly post treatment and showed an increase at recurrent disease (A, C, E), whereas the viral load in whole blood (gray line) was in general low (A, C, E). The dashed lines show the COV for the viral load in brush (black) and in blood (gray). The dynamics in normalized IgA antibody responses in serum of patients against EBNA1 (black line) and VCA-p18 (gray line) showed a divers pattern in the patients (B, D, F). In the dashed line the COV of 1.0 for serology is indicated.

The third example is a patient who had progressive disease despite treatment. The duration of treatment was longer than the protocol would prescribe which was due to a long waiting time for radiotherapy. Therefore chemotherapy was given before radiotherapy. Under these circumstances the development of a distant metastasis was possible despite treatment. The viral DNA load in the brush was not as extreme as in the other 2 patients, although

a large decrease in viral DNA load was observed during treatment. The distant metastasis developed before the viral DNA load in the brush increased. In the circulation viral DNA load was only slightly elevated just after the appearance of the metastasis. The antibody responses were very low in this patient. After the detection of the metastasis EBNA1 levels constantly increased whereas the VCA-p18 levels remained stable.

These 3 examples of the kinetics of viral biomarkers indicated that not the level itself but the variations in time may be indicative for prediction of clinical behavior and treatment success. This was mainly observed with the level of EBV DNA in the brush, since viral DNA load in the circulation was mostly low or undetectable. The serological response was more stable and treatment success was not paralleled with rapid decline of the EBV antibody levels.

Dynamics in viral biomarkers in patients developing local recurrence

Since the viral DNA load in the brush is considered to only reflect local tumor activity, 2 patients who developed local recurrences were analyzed in time for the 4 viral biomarkers (Fig. 3). The first patient did not have high levels of EBV DNA in the brush, but showed a decline in viral DNA load during treatment to undetectable levels. Thirty weeks post treatment the viral DNA load increased to levels above COV value. In week 60 a local recurrence was detected (arrow). The viral load in the circulation could not be measured often and remained negative in follow up. Antibody levels to EBNA1 and VCA-p18 had a similar trend that did not reflect the tumor decrease during treatment. At the moment of the local recurrence an increase in antibody levels was not observed, which might be due to irregular sampling in addition to slow kinetics of antibody responses.

The second patient had fluctuations in the viral DNA load in the brush during treatment. At the time of local recurrence viral DNA load had raised and blood levels of EBV DNA were low but detectable. The serological pattern was more dynamic than in the other patients and VCA-p18 antibody levels decreased during initial treatment and increased before the local recurrence was detected whereas IgA-EBNA1 remained stable.

Local recurrences might be detected by analyzing viral DNA load in the brush where the COV value could be used as a warning signal of unstable tumor activity. However the viral DNA load in the circulation as well as the serology could not clearly predict the development of a local NPC recurrence in these patients.

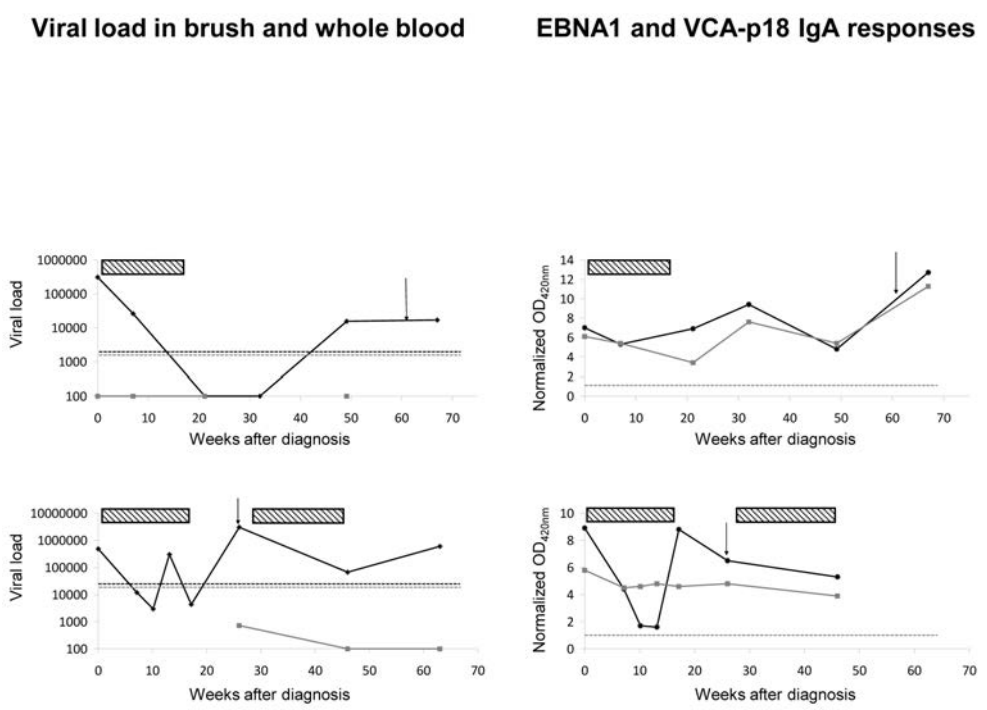

Figure 3 | Dynamics of EBV markers at NPC recurrence are presented. Two typical examples are shown of patients having recurrent disease. The dashed bar indicates the period of therapy. The amount of EBV genomes detected in tumor cells derived by NP brushing (black line) showed an increase at recurrent disease (A, C), whereas the viral load in whole blood (gray line) was in general low (A, C). The dashed lines show the COV for the viral load in brush (black) and in blood (gray). The dynamics in IgA antibody responses in serum of patients against EBNA1 (black line) and VCA-p18 (gray line) showed an increase at the recurrence in the patient presented in B, whereas the patient presented in D did not show a increase in IgA responses. In the dashed line the COV of 1.0 for serology is indicated.

Prediction of prognosis by the level of viral biomarkers at diagnosis and post treatment

The levels of the 4 viral biomarkers were analysed for prediction of prognosis (Fig. 4). Survival was analyzed by grouping the patients based on the COV. IgA antibody levels against either VCAp18 or EBNA1 at diagnosis did not predict prognosis. The viral DNA load in brush was above COV in most samples, which made survival analysis for this biomarker not informative. The viral load in the circulation at diagnosis did not relate to prognosis.

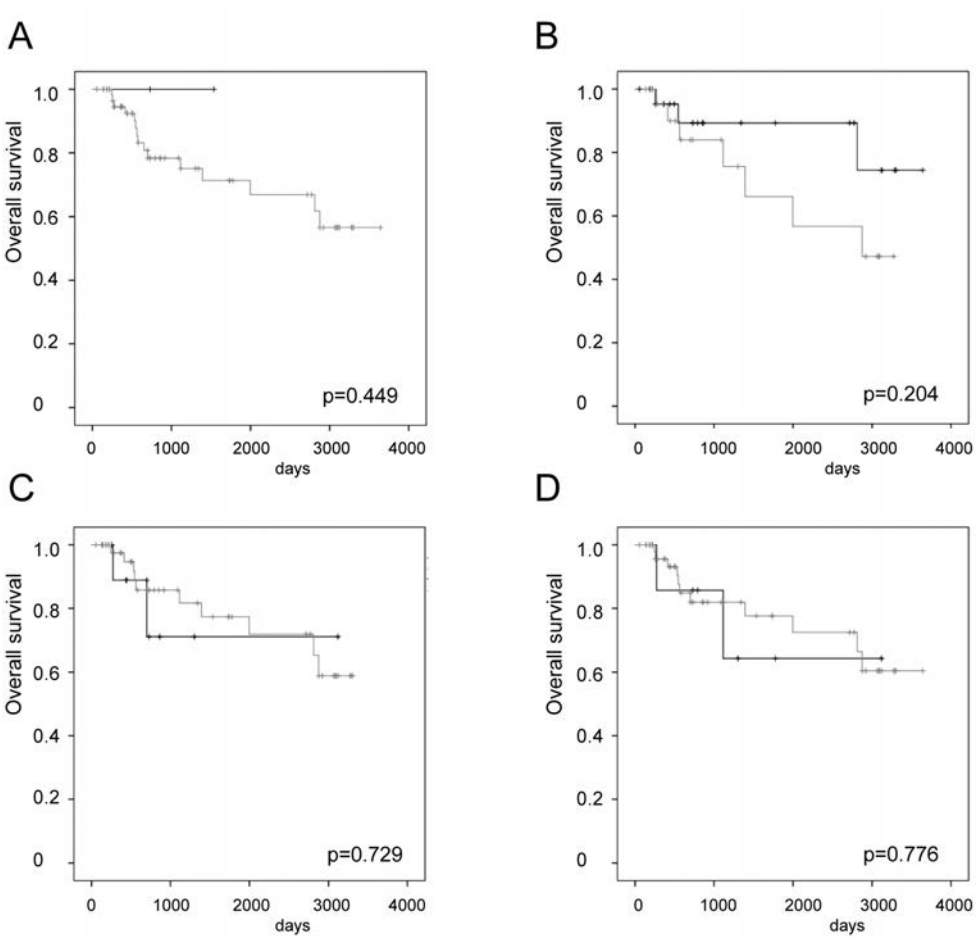

Figure 4 | Survival analysis based on COV of 4 EBV makers at diagnosis. Viral load in NP brush (COV=2,300 EBV copies per brush) (A) and in circulation (COV=2,000 EBV copies per ml whole blood) (B), IgA responses (COV=1) against VCA-p18 (C) and EBNA1 (D).

The Kaplan-Meier survival curves using the level of the viral biomarkers 2 months post treatment showed in the levels of EBV antibodies a difference in survival. Patients with antibody levels below COV at 2 months post-therapy showed a better prognosis. Both the antibody levels to VCA-p18 as EBNA1 showed a difference in prognosis (p-value respectively 0.042 and 0.041). The levels of viral load in both NP brush and whole blood did not relate with prognosis, indicating that the viral DNA load in the brush above COV was not suitable as predictor of prognosis, because only few patients remained truly below COV (Fig. 5A). The viral DNA load in blood was negative in most samples and therefore could not discriminate for prognosis (Fig 5B).

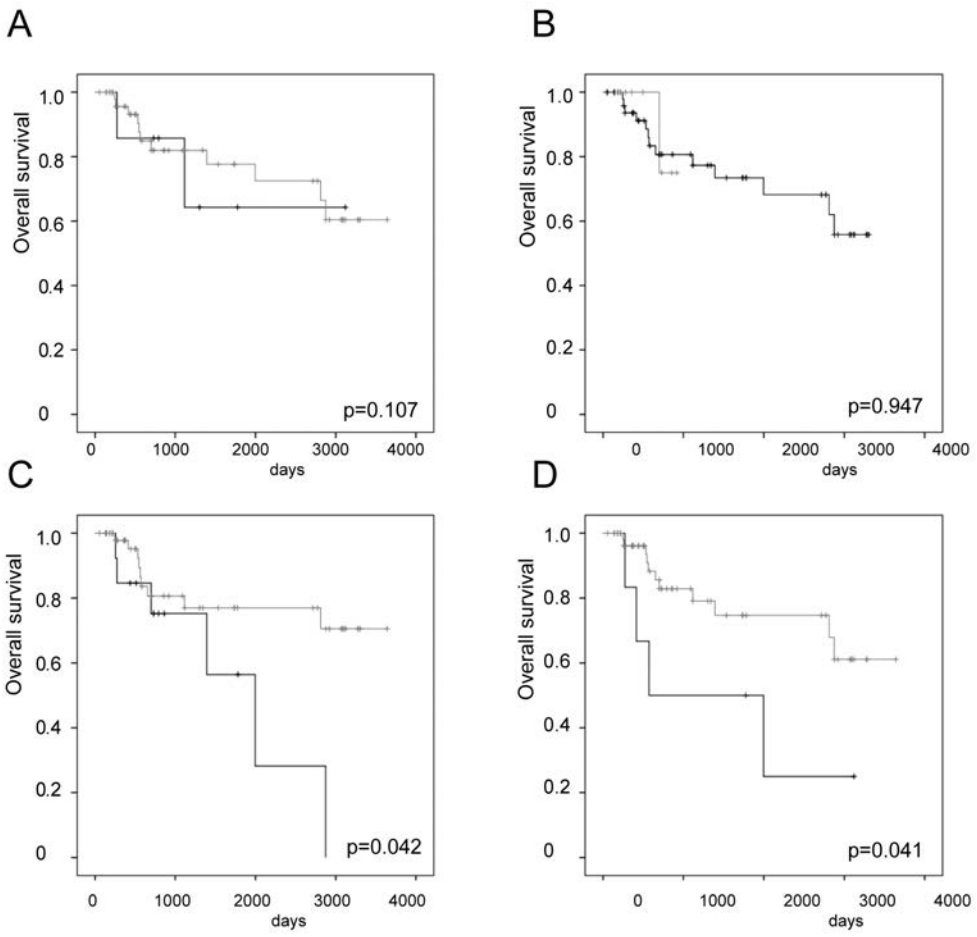

Figure 5 | Survival analysis based on COV of 4 EBV makers at 2 months post treatment (pt). Viral load in NP brush (COV=2,300 EBV copies per brush) (A) and in circulation (COV=2,000 EBV copies per ml whole blood) (B), IgA responses (COV=1) against VCA-p18 (C) and EBNA1 (D).

DISCUSSION

NPC is a complex disease caused by an interaction of EBV infection, environmental factors inducing genetic aberrations and (epi)genetic dysregulation of host genes, in a multi-step process of carcinogenesis[41]. Presence of EBV in all tumor cells makes EBV a good diagnostic target for NPC. The presence of aberrant antibody response to EBV antigens is a diagnostic hallmark for NPC presence and can be used for risk screening, diagnosis and prognosis[24,25,28,42,43]. The detection of fragmented EBV DNA in plasma or serum from

patients with NPC has been described as an additional valuable marker for the diagnosis and monitoring of NPC[9,11,44]. This study revealed that measuring EBV DNA in whole blood did not reach sufficient sensitivity to serve as diagnostic tool for detecting NPC, nor for defining metastatic disease, mainly because many samples remained below COV, which is distinctly different from experiences in patients with EBV-related lymphoproliferative diseases[38,45]. On the other hand, the direct measurement of tumor cells by EBV DNA load PCR in non-invasive NP brushings might be useful since changes in viral load rather than their presence above COV were indicative of treatment response and predictive for the development of tumor relapse. EBV-IgA serology proved less useful for post-treatment monitoring, since antibody levels remained high in most cases.

In Indonesia, NPC is by far the most prevalent head and neck tumor consisting of 28% of all ENT tumors in our hospital[1]. Although NPC is highly chemo and radiosensitive, the 5 year survival rates in Indonesia are rather low. Studies from Yogyakarta and Jakarta have shown a 3-year survival of less than 30% for adults and 5-year survival around 7-19% in juveniles, mainly due to late presentation of patients, poor treatment facilities and protocol compliance and therefore resulting in low treatment success[46] (Adham et al., manuscript submitted). In this study only 1 of the 68 patients was diagnosed for early stage NPC. The majority of patients (47/68; 69%) received neoadjuvant chemotherapy followed by conventional or hyperfractionated radiotherapy. Hyperfractionation was given to 30 patients (44%), with the doses of 70 Gy. Due to the low compliance of 70 Gy doses by most of our patients, treatment was changed to concurrent chemoradiation with doses of 40-60 Gy weekly, reaching the same dose in total. Concurrent chemoradiotherapy plus adjuvant chemotherapy was described to result in a better survival as analyzed in 508 patients with NPC[47]. Despite the improvement of treatment approach many of our patients showed a relapse of NPC, most probably relating to delays in treatment time and irregular treatment intervals, similar to the Yogyakarta experience[46].

EBV-related parameters as biomarker could contribute in the prediction of recurrent disease. Measuring EBV DNA load in NP brushings provides a highly specific tool for primary NPC diagnosis with minimal discomfort, giving better sensitivity and specificity compared to EBV-IgA serology and EBV DNA load in blood[1,24].

Previous studies detected a high incidence of detectable EBV DNA in the plasma and serum of NPC patients, and revealed that the plasma EBV DNA level provided a tumor marker that could be used in staging and prognosis of NPC[7–9,27,29,48]. In whole blood samples with high EBV-DNA load, no viral (BARF1) mRNA could be detected, excluding the possible presence of circulating intact NPC tumor cells[17]. Because plasma EBV-DNA is highly fragmented, it is considered to derive from apoptotic fragments released by the NPC tumor[14]. Our result

showed that in circulating whole blood, viral DNA load was low at diagnosis being detectable in only 49% of patients, which was not expected but is in agreement with previous independent studies using whole blood from NPC patients[18,49].

Viral load in swabs and in the circulation of the patients was described to contribute in the follow up of the patients post treatment[9,27,50–52]. In our study a significant decrease in viral load in brush and whole blood was observed after treatment (respectively p=0.001 and p=0.005), which is in agreement with previous studies[23,49,53], suggesting viral load could reflect disease activity and may be used to monitor tumor relapse. The presence and level of EBV DNA in plasma is suggested to represent an important and sensitive index in diagnosing the residual tumor activity and relapse of NPC[44,54,55]. The clearance of EBV DNA in the blood during and after treatment could indicate successful treatment[13]. Serial measurement of plasma EBV DNA levels during radiotherapy or after surgical resection for recurrent NPC showed initial increase followed by a rapid decline (139 min) in plasma EBV DNA concentration[9,52].

Persistent increase in plasma DNA levels may associate with incomplete eradication of tumor cells therefore indicate poor survival[9,27,56–58] . The monitoring of plasma levels is sensitive and highly specific in detecting disease recurrence and metastases, which must be attributed to an enormous load of tumor cells releasing viral DNA in blood while replicating[59,60]. Chan et al. showed that plasma EBV DNA analysis may be useful for detecting early NPC in individuals without clinical suspicious of NPC[61]. In the study presented here tumor reduction during treatment was reflected by dramatic decreases (median 300-fold) in viral load in the brush and in whole blood resulting in nearly undetectable levels. However, just before relapse viral load in the circulation was not highly elevated (Figure 4). Viral load in brush was slightly elevated in 3 of 4 cases with local recurrence but is not suitable for the detection of distant metastasis, since viral load in brush only reflects carcinoma specific EBV involvement at the anatomical site of tumor development[18]. Hao et al showed promising results with nasopharyngeal swabs analyzed for viral load via EBV LMP-1 DNA detection which could detect very early recurrence even after radiation therapy with sensitivity 91.7% and specificity of 98.6%[53]. Our study showed promising results in this respect, but was not nearly as good predictive as the Hao study.

Antibody levels to EBV-VCA may serve as potential diagnostic marker of NPC, with IgA-VCA having clear advantage over IgG-VCA[62]. From 20 years follow-up Cao et al. mentioned ascending titres IgA-VCA and IgA-EA strongly related with an increased risk for NPC[63], confirming prior work of Ji et al.[43]. In our study, antibody levels against VCA-p18 and EBNA1 were elevated above COV values in all patients at diagnosis confirming previous results that serology is diagnostically useful[28,29,64–66]. A case report from a Dutch female NPC patient recently clearly demonstrated the potential value of EBV-IgA serological monitoring during

follow-up, with decreasing reactivity post treatment and increasing responses detectable at 6 month prior to clinically apparent relapsing disease. This however could not be reproduced in the cohort of NPC patients in this study. The prognostic value of EBV-IgA markers seems limited for the Indonesian population, as these markers showed little fluctuation during follow-up. This may however relate to suboptimal treatment, allowing low level tumor (and related EBV activity) persistence, with inherent risk for relapsing disease.

High titers antibodies to VCA and early antigen, especially of high IgA class, or high titers that persist post treatment, were found to be associated with a poorer prognosis[42,67]. Our results showed that serology did not predict for survival. Serology cannot be used alone as a marker of malignancy, but could be useful as an initial indicator in a screening/monitoring program, in adjunct to other markers and diagnostic procedures and early diagnosis and for monitor patients post treatment[63,68–70]. Although the dynamics of the antibody responses were not indicative for treatment response nor for development of recurrent disease, the level of antibodies did reflect the prognosis of the patient. High levels of VCA-p18 or EBNA1 showed a better prognosis therefore high levels of EBV antibodies could be protective. Following treatment and increased exposure to viral antigens, the immune system might be more prone to detect residual NPC tumor cells, which is beneficial for patients with NPC on the long term (Fig.5). This result was not observed at 2 months post treatment, since this protective role of immune system might be camouflaged by the strong treatment effect. However at 2 years post treatment the effect was significant ($p<0.05$) since antibody levels of IgA VCA-p18 and EBNA1 were above COV in more disease free patients. The Kaplan-Meier curve might indicate that presence of high antibody levels remaining post treatment might have a protective role and results in a longer survival. As reflected in table 3, 2 years post treatment the IgA antibody level to VCA-p18 and EBNA1 above COV is related to disease free status.

EBV could be used as marker for the diagnosis of NPC, monitoring treatment response and prognosis. Viral load in NP-brushings combined with positive IgA antibody responses is suitable for the reliable detection of NPC. Tumor reduction is clearly reflected by decreases in viral load in both brush and blood. However the prediction of recurrent disease is more difficult. Viral load in brush indicates tumor activity in the nasopharyngeal region and could be useful for local recurrence, but dynamics analysis in time is preferred above static measurements. For better understanding of the viral markers a study should be conducted in which regular sampling is performed and treatment could be given in a standard scheme, which proved rather difficult in the current Indonesian situation.

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Chapter 6

Current status of cancer care for young patients with nasopharyngeal carcinoma in Jakarta, Indonesia

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ABSTRACT

Background: Nasopharyngeal carcinoma (NPC) is endemic in Indonesia and 20% of the patients are diagnosed before the age of 31. This study evaluates presentation and treatment outcome of young patients in Jakarta, in a tertiary referral centre.

Methods: Forty-nine patients under the age of 31, diagnosed with NPC between July 2004 and January 2007, were evaluated. Baseline data included histological type, stage of disease and presenting symptoms. We intended to follow all patients after diagnosis to reveal treatment outcome and overall survival (OS).

Results: All but two patients had advanced stage disease (94%), 7 (14%) had distant metastasis. The median interval between start of complaints and diagnosis was 9 months. Forty-two patients were planned for curative intent treatment. Eleven patients (26%) never started treatment, 2 patients did not complete treatment and 3 patients did not return after finishing treatment. Four patients died before radiation could start. Three patients died within 4 months after treatment. Nine patients (21%) had a complete response.

Due to the high number of patients who were lost to follow-up (LFU), OS was analyzed as follows: a best-case (patients censored at last contact) and a worst-case scenario (assuming that patients who did not finish treatment or had disease at last contact would have died). The 2-year OS for patients without distant metastases was 39-71%.

Conclusion: Treatment outcome for young patients with NPC in this institute was poor. Improvement can be achieved when NPC is diagnosed at an earlier stage and when there is better treatment compliance.

BACKGROUND

The incidence of nasopharyngeal carcinoma (NPC) in Indonesia is estimated to be 6:100.000, meaning that every month at least 1000 patients are diagnosed. Probably related to better diagnostics and improved awareness this number increases every year[1]. In Jakarta 20% of the patients are diagnosed before the age of 31[1]. A study conducted in Yogyakarta, revealed a 3-year overall survival of 30% for adults with NPC, compared to 80% in literature[2,3]. The current study reveals the presentation and treatment outcome of young patients in Jakarta, in a tertiary referral centre.

NPC in young patients differs in certain aspects from adults. The percentage of non-keratinizing undifferentiated carcinoma is higher, and the association with Epstein-Barr virus (EBV) is stronger[1,4-6]. Young patients have more advanced disease at diagnosis and distant metastases are more frequently seen[4-9]. This might be caused by the undifferentiated state of the tumor, which is prone to develop distant metastasis[4,6-7]. Another hypothesis is the late recognition of complaints belonging to NPC in young patients, since early symptoms of NPC are non-specific and can look like ordinary upper airway infections, which are common in children.

Treatment for young patients generally follows the guidelines established for adults; radiotherapy on the nasopharynx and cervical nodal levels, usually combined with chemotherapy[4]. Despite the advanced stage at presentation, survival of young patients does not differ from adults. Several retrospective trials have proven the benefit of additional chemotherapy in juveniles[4,9-12]. Five-year disease-free survival varies between 45-77% and 5-year overall survival is 52-77%[5,7-12]. Recently, Buehren et al. published more promising results by adding adjuvant interferon beta after standard (chemo-) radiotherapy. This resulted in an event-free survival rate of 92.4% after a median follow-up of 30 months and an overall survival of 97.1%[7,13].

All these results are derived from top-end hospitals and some are in clinical trial settings. Here we present a prospective observational study on routine treatment results of young patients with NPC at a top end hospital in Jakarta. We will describe the tumor characteristics and complaints at presentation, the given treatment and the treatment outcome.

METHODS

Patients

This was a prospective cohort study. All patients diagnosed with NPC between July 2004 and January 2007 at the Rumah Sakit Cipto Mangunkusumo (RSCM), a university hospital in Jakarta, were eligible for inclusion. Patients were included if they were below the age of

31 at diagnosis and had histological proven NPC. In this period 228 patients were diagnosed with NPC, and 49 patients met the inclusion criteria of age and histological confirmed NPC. Ethical approval was obtained at the Ethical committee of the Faculty of Medicine of the university of Indonesia. All patients or their parents/ legal guardian signed informed consent. To get more insight in the specific problems of patients at young age, the patients were divided into two groups, i.e. ≤15 years and >15-30 years.

Baseline information consisted of patient demographics, including the type of insurance. Jakarta has three types of insurances; jamkesmas (poor people), askes (civil servants) and patients who pay health care out of the pocket or have private insurance (self-finance)[14-15]. We hypothesized that type of insurance would have an impact on treatment outcome.

Presentation and Diagnosis

Information on symptoms was gathered from the clinical medical record. Symptoms were scored for presence at diagnosis and duration till diagnosis. The histological diagnosis was made according to the World Health Organization (WHO) classification, WHO type 1; keratinizing squamous cell carcinoma, WHO type 2; non-keratinizing squamous cell carcinoma, WHO type 3; undifferentiated carcinoma. The extent of disease was determined by clinical examination using rigid or flexible nasopharyngoscopy, Computed Tomography (CT)-scan of the head and neck region, chest radiography, ultrasonography of the abdomen and a bone scan. Tumor stage was classified according to the 2002 criteria of the 6th American Joint Committee on Cancer (AJCC).

Treatment

Due to the waiting time to radiation, different schedules were used. Three different radiation schedules were used; conventional fractionated schedule (daily fraction of 2 Gy, total 33-35 fractions); hyper fractionated schedule (2 fractions/ day of 1.2 Gy with 6 hours in between, total dose 81.6 Gy); accelerated hyper fractionated schedule (daily fraction of 1.8 Gy in the first 4 weeks, followed by 2 weeks of daily 1 fraction of 1.8 Gy and a surdosage to macroscopic tumor of 1.5 Gy with 6 hours in between, total 72 Gy). All schedules could be completed in 6-7 weeks. Neo-adjuvant chemotherapy consisted of intravenous cisplatin 100 mg/m2/day on day 1, and 5-fluoro-uracil 1000 mg/m2/day on day 1-5, every 3 weeks for 3-4 courses. Concurrent chemotherapy consisted of intravenous cisplatin 40mg/m2 weekly during radiotherapy.

Patients with distant metastasis received palliative chemotherapy; cisplatin 100 mg/m2/day on day 1 and, 5-fluoro-urasil 1000 mg/m2/day on day 1-4. The number of courses depended on the clinical condition. Palliative radiotherapy was given on bone metastasis.

Follow-up

Patients were scheduled for routine follow-up at the outpatient clinic. Treatment response measurements were planned 8 to 12 weeks after treatment, by physical examination, nasopharyngoscopy and CT-scan. The follow-up schedule proceeds with 3 monthly visits during the first 2 years after radiotherapy.

Statistics

To test for association between age and tumor stage at diagnosis, linear-by-linear test was used. For symptoms at diagnosis two scales were constructed: the number of complaints at diagnosis and the maximum duration to diagnosis. For patient's missing data on symptom duration, the median duration was imputed. Associations between these two scales and both age (as a continuous variable) and AJCC stage were tested using linear-by-linear tests.

Association between age and diagnosis-to-treatment interval (DTI) and overall-radiotherapy-treatment time (OTT) was assessed by Spearman correlation test. The Statistical Package for the Social Sciences, version 20 was used for analysis. P-values less than 0.05 were considered as significant.

Kaplan-Meier analyzed overall survival. Survival time was defined as the time between the date of diagnosis till the date of death. Stratification was done by M stage, and M0 was further stratified by age (0-15 and 16-30). For comparison between the 0-15 and the 16-30 age group a log rank test was used.

RESULTS

Patients

Forty-nine patients were included. The median age was 21 and ranged between 3-30 years. WHO type 3 was the histological type in 46 patients (94%) and 3 patients (6%) had WHO type 1. The mean follow-up period for the patients without distant metastasis at diagnosis was 18 months and for patients with distant metastasis 7 months.

Stage of disease at presentation

T-stage was dominated by advanced stage (66%). Lymph node metastasis was seen in 96% of the patients (table 1). Ninety-four per cent had advanced stage of disease. Seven patients (14%) had distant metastasis at diagnosis. All had metastases to the bone. In addition, two

patients had lung metastasis and one of these had also liver metastasis. No association was found between age and stage of disease at presentation (linear-by-linear p=0.85).

Table 1 | Patient & tumor characteristics

		0-15 Year n = 14	16-30 Year n = 35
AGE AT DIAGNOSIS			
	Median	11	26
	(Range)	(3 - 15)	(16 - 30)
GENDER		Number (percentage)	Number (percentage)
	Male	10 (71)	18 (51)
	Female	4 (29)	17 (49)
INSURANCE			
	Jamkesmas	14 (100)	27 (77)
	Askes	0 (0)	2 (6)
	Self Finance	0 (0)	4 (11)
	Missing		2 (6)
TUMOR			
T	T1	0 (0)	2 (6)
	T2a	0 (0)	2 (6)
	T2b	4 (29)	9 (26)
	T3	5 (36)	8 (23)
	T4	5 (36)	14 (40)
N	N0	0 (0)	2 (6)
	N1	0 (0)	7 (20)
	N2	3 (21)	7 (20)
	N3a	8 (57)	15 (43)
	N3b	3 (21)	4 (11)
M	M0	12 (86)	30 (86)
	M1	2 (14)	5 (14)
STAGE	2b	0 (0)	3 (9)
	3	3 (21)	4 (11)
	4a	0 (7)	9 (26)
	4b	9 (64)	14 (40)
	4c	2 (14)	5 (14)

Symptoms at diagnosis

Information on presenting symptoms at diagnosis was available for 41 patients (table 2). The median number of complaints at diagnosis was 5 (range 2-10). The median interval to diagnosis was 9 months (range 1-36 months). A neck mass was mentioned in 93% of the patients at diagnosis, more than 50% of the patients (21/40) had bilateral neck masses.

No associations (linear-by-linear) were detected between either age and the number of complains at diagnosis (p= 0.41) or the duration to diagnosis (p= 0.79). Also no associations

were found between the stage of disease at diagnosis and the number of complaints (p= 0.25) and interval to diagnosis (p=0.29).

Table 2 | Complaints at diagnosis and interval between first appearance and diagnosis.

	0-15 Year n= 10 (100%)	16-30 Year n= 31 (100%)		0-15 Year Duration of symptom in months	16-30 Year Duration of symptom in months
NECK MASS					
Yes	10 (100)	28 (90)	Median	11	9
No	0 (0)	2 (6)	Range	5-18	2-36
Missing		1 (3)			
NASAL CONGESTION					
Yes	8 (80)	23 (74)	Median	4	3
No	2 (20)	8 (26)	Range	1-18	1-12
			Missing		1
EPISTAXIS					
Yes	7 (70)	17 (55)	Median	4	2
No	3 (30)	14 (45)	Range	1-18	1-12
			Missing		1
POST NASAL DRIP					
Yes	4 (40)	10 (32)	Median	7	10
No	6 (60)	17 (55)	Range	4-10	3-12
Missing		4 (13)	Missing	2	4
DIPLOPIA					
Yes	3 (30)	9 (29)	Median	1	3
No	7 (70)	22 (71)	Range	0.5-5	0.25-7
DEAFNESS					
Yes	7 (70)	21 (68)	Median	1	1
No	3 (30)	10 (32)	Range	1-2	1-2
			Missing		1
TINNITUS					
Yes	5 (50)	17 (54)	Median	3	3
No	5 (50)	14 (45)	Range	2-12	1-24
			Missing		2
EAR PAIN					
Yes	2 (20)	7 (23)	Median	2	1.5
No	6 (60)	20 (65)	Range	2-2	0.03-12
Missing	2 (20)	4 (13)	Missing	1	3
OTORRHEA					
Yes	0 (0)	3 (10)	Median		6
No	9 (90)	22 (71)	Range		6-12
Missing	1 (10)	6 (19)			
CEPHALGIA					
Yes	5 (50)	21 (68)	Median	6	3
No	5 (50)	10 (32)	Range	1-12	1-36
			Missing		2
NERVE PARALYSIS					
Yes	2 (20)	5 (16)	Median	5.5	1
No	7 (70)	25 (81)	Range	5-6	1-5
Missing	1 (10)	1 (3)	Missing		1

Treatment

Forty-two patients could be planned for treatment with curative intent. For 22 patients data was available on given radiotherapy treatment (table 3). The median interval between diagnosis and radiotherapy was 110 days (28-690 days). Patients in the 16-30 age group had to wait longer than the younger patients (130 vs. 77 days), although no association with age was found (Spearman correlation p=0.99).

Table 3: Radiotherapy treatment

		0-15 Year	16-30 Year
DIAGNOSIS TO RADIOTHERAPY IN DAYS		n=7	n=15
	Median	77	130
	(Range)	(28 - 690)	(34 - 320)
RADIOTHERAPY DURATION IN DAYS OVERALL		n=4	n=14
	Median	55	56
	(Range)	(50 - 160)	(38 - 77)

For 18 patients data on the overall radiotherapy treatment time (OTT) was available. The median OTT was 55 days (range 38-160), no association with age was found (spearman correlation p= 0.41). Since almost all patients had jamkesmas insurance, no association between the insurance type and the DTI or OTT could be found.

Treatment outcome and follow-up

Directly after diagnosis 11 patients (26%) did not return to the hospital. Two patients stopped therapy during treatment, and directly after therapy 3 patients never returned to the hospital. Despite several attempts to contact these 16 patients, no information on their health status could be retrieved. Four patients died before radiation treatment could start. Figure 1 shows the chart-flow.

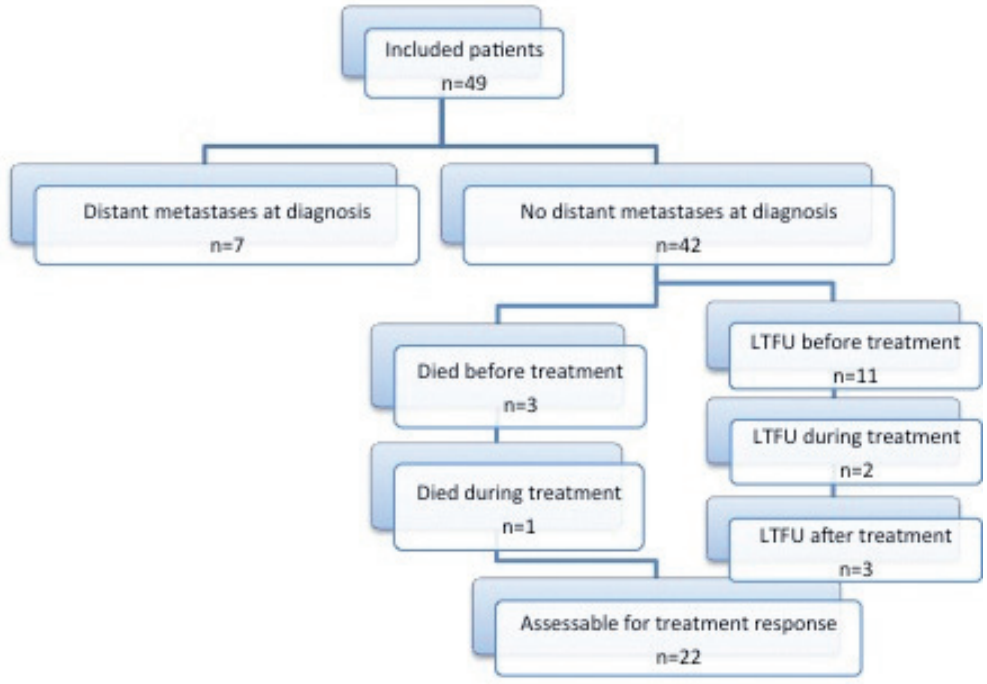

Figure 1 | Patient overview.
LTFU= lost to follow-up.

Accordingly, for 22 patients the effect of treatment could be studied. Three patients died within 4 months after radiotherapy, before response was assessed. For three patients survival data was available, but no data on therapy response; one died 19 months after neo-adjuvant chemotherapy (unknown if he finished radiation treatment), one was alive 30 months after radiation and clinical suspect for distant metastases, and one died 65 months after radiation (reason unknown).

Nine patients underwent examination 2-3 months after treatment, one patient had the examination 1 month after treatment and six patients had examination later than 3 months after therapy. Complete response was seen in 9 of these 16 patients, partial response in 5 patients and progressive disease in 2 patients.

Overall survival

The number of patients who were lost to follow-up (LFU) in this study was high. Despite multiple attempts to contact them or their family, it was not possible to minimize the missing data. The assumption that the risk to death was equally distributed between patients who

were LFU and patients who were still in the study is not likely. This is based on the fact that some patients never started treatment, stopped during treatment or had disease at the last date of follow-up. Therefore we made two Kaplan-Meier curves, representing a best-case scenario and a worst-case scenario. The best-case scenario is a regular Kaplan-Meier curve, wherein all patients are censored on the last date of follow-up. For the worst-case scenario; for patients without distant metastasis, all patients who did not return to the hospital before starting treatment (n=11), before finishing treatment (n=2), or who had disease at last moment of contact (n=6) were assumed to be death at the last date of contact; for patients with distant metastasis at diagnosis, the last date of contact was set as the date of death. A realistic overall survival curve will be positioned between these two Kaplan-Meier curves.

The 2-year overall survival for patients without distant metastasis at diagnosis was 39-71% (worst- and best-case scenario, respectively). The 2-year survival for patients with distant metastasis at diagnosis was 0% (table 4). Overall survival, analyzed in the best-case scenario was significantly poorer for the younger patients (log rank p= 0.021). In the worst-case scenario this was not significant (log rank p=0.142)(figure 2 and 3).

Table 4: Overall survival

	6 months		2 years		5 years	
	Worst-case scenario	**Best-case scenario**	**Worst-case scenario**	**Best-case scenario**	**Worst-case scenario**	**Best-case scenario**
0-15 year (M0, n=12)	58%	78%	20%	50%	0%	0%
16-30 year (M0, n=30)	60%	91%	46%	79%	23%	52%
0-30 year (M0, n=42)	60%	87%	39%	71%	16%	38%
Distant metastasis (n=7)	43%	83%	0%	0%	0%	0%

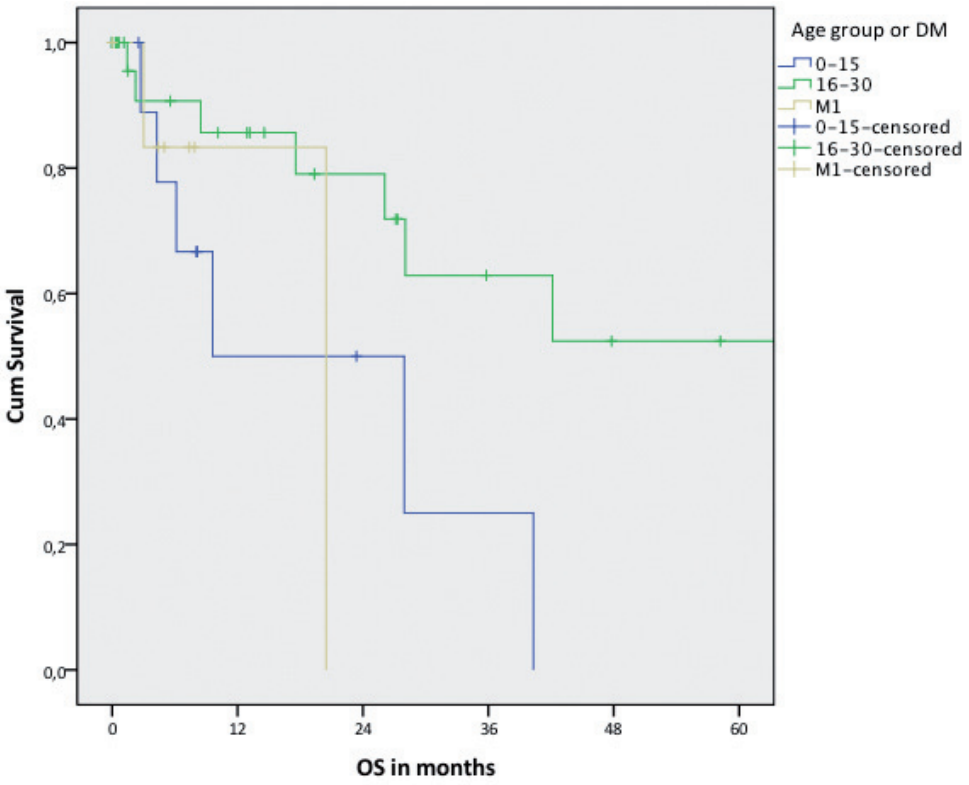

Figure 2 | Overall survival: best-case scenario. All patients who were lost to follow up were censored at the moment of last contact (Log rank is p= 0.021, when comparing patients without distant metastasis: 0-15 vs. 16-30 year).

DM = distant metastasis at diagnosis; OS = overall survival

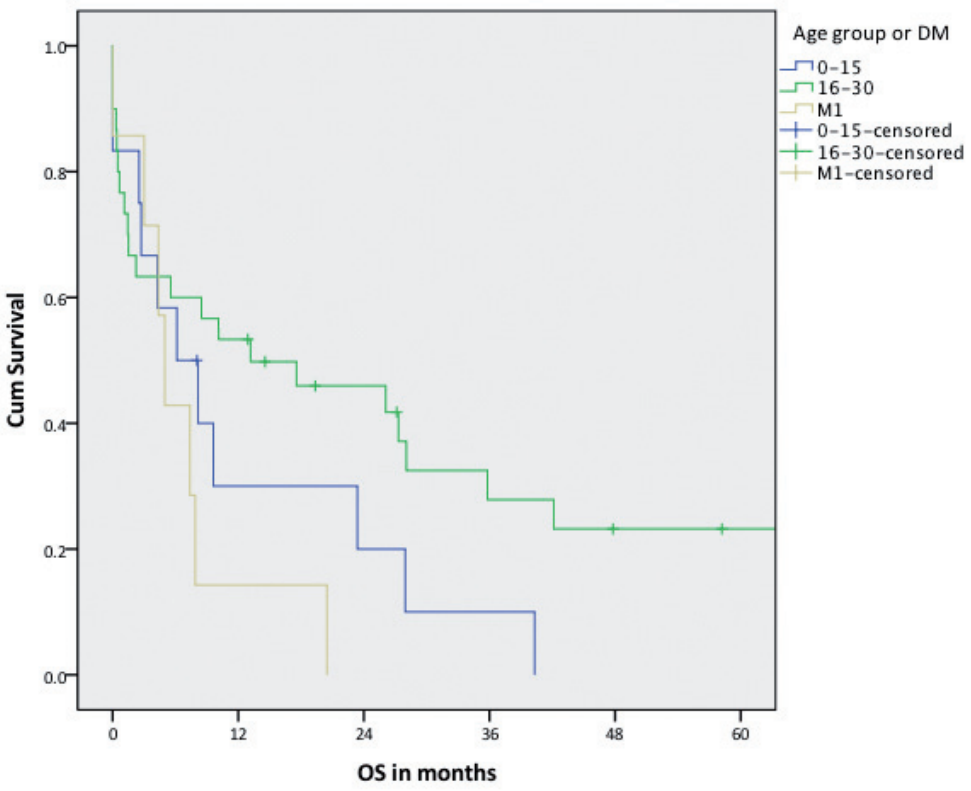

Figure 3 | Overall survival: worst-case scenario. All patients who were lost to follow up before treatment (n=11) or during treatment (n=2), or who had disease at last moment of follow up (n=6) are assumed to be death (Log rank p= 0. 142, when comparing patients without distant metastasis: 0-15 vs. 16-30 year).

DM = distant metastasis at diagnosis; OS = overall survival

Overall survival was tested on association with stage of disease, symptoms at diagnosis, waiting time for radiotherapy and treatment duration. No significant results were found. It was not possible to test for association with insurance, since the group of patients with other insurance than jamkesmas was too small.

DISCUSSION

Cancer is the leading cause of death worldwide[16-17]. The distribution of cancer mortality shifts towards the low- and middle-income countries. Currently, 70% of cancer deaths occur in these countries and this burden increases every year[16-18]. Their health-care systems are not prepared for the number of patients. Unlike high-income countries, where cancer survival

improves due to better treatment facilities and enhanced protocols, low-income countries lack facilities and medication. Funding for research and solutions aiming to resolve these limitations is hardly available. The gap in treatment results between high-income and low-income countries is therefore widening[17]. Major improvement in the health care systems is needed. Although many authors have emphasized this, solid data on the actual problems are lacking. This study reveals some of the current problems in the treatment of NPC in Jakarta, a major referral hospital and one of the top end hospitals in Indonesia.

Cancer care for young patients with NPC in Jakarta is poor compared to international literature. In literature, 1-4% of the young patients with NPC have distant metastasis at initial diagnosis[4-5]. In this study 14% of the patients presented with distant metastasis. Two years after diagnosis many patients were lost to follow-up, only 47 per cent of them were still in the study (23/49). Ten out of these 23 patients had already died at this point. The 5-year overall survival for patients without distant metastasis lies between 16-38%, compared to 52-77% in the literature[4-5,7-13]. These results might be caused by the late stage of presentation at the hospital, insufficient treatment (compliance) and poor follow-up.

Advanced stage of disease at diagnosis was seen in 94% of the patients. Since stage of disease is strongly associated with prognosis, this partly accounts for the poor survival. Advanced stage at diagnosis is related to a long interval to diagnosis[19]. In our study the mean interval from start of complaints till diagnosis was 9 months, which is long compared to the 4 to 8 months found in China[19], India[12] and Turkey[5]. This long interval can be caused both by patient's or doctor's delay. Early stage symptoms of NPC look like an ordinary inflammatory upper airway infection. In our young patient group, the early stage symptoms are not mentioned as complaints with the longest duration. Apparently, the early symptoms are not evidently present or do not trigger patients to seek medical help. The latter explanation might be plausible in this patient group, due to the non-specificity of the complaints and the frequency of upper airway complaints in the young population.

Neck masses, a late stage symptom, were present in 93 per cent of our patients at diagnosis. In almost all patients this complaint existed with the longest interval to diagnosis. One should assume that when a neck mass is present, a patient (or parent) should make effort to consult a doctor. Instead, a time interval of 9.5 months was found before definitive diagnosis. It seems that patients (and probably doctors) are not aware of the probability for NPC involvement in young patients with an unexplained neck mass.

Patient's delay to diagnosis can also be caused by the long distance to health care facilities or limited financial resources of patients, 84% had poor men's insurance. Besides, we know by experience that many patients first seek medical help in the alternative circuit. Even when NPC is diagnosed some patients prefer alternative therapy above conventional. We cannot confirm this by our study results, but eleven patients did not return to the hospital after diagnosis. Unfortunately, we could not retrieve the reason for not returning. More public awareness about the symptoms of NPC and need for early treatment with (chemo-) radiotherapy can contribute to an earlier consultation of the doctor and better compliance to the advised therapy. Previous studies have already shown the effectiveness of public awareness campaigns in breast and cervical cancer[20].

As mentioned before, the doctor can also cause the delay to diagnosis; when doctors do not recognize the symptoms as related to cancer or when they are not aware of the high probability of NPC. Earlier research revealed that the knowledge of general practitioners (GP's) on NPC in Indonesia was insufficient[21]. A sequel study showed that after teaching there was a great improvement of knowledge[22]. More educational programs can improve early diagnosis. Furthermore, with the increasing awareness of NPC's associated with Epstein-Barr virus (EBV) infection and the availability of tests with EBV-related tumor markers which can be performed by the GPs, improvement in earlier diagnosis is within reach[1,23-25].

Another result of this study was the insufficiency of the treatment itself. The median interval between diagnosis and radiotherapy was almost 4 months. This is partly caused by the insurance system. Almost all patients had jamkesmas insurance. Hereby, approval is needed for every investigation and treatment, which takes valuable time. Another key reason for the long interval to treatment is shortage in capacity of radiotherapy facilities. In 2008, 35 radiotherapy devices were available for a population of 229 million. A substantial number of these devices are out of order on a regular base, resulting in 0.13 accelerators per million inhabitants[26]. This is not enough. For comparison, in Europe 2- 5.5 accelerators are available per one million inhabitants[27]. We assumed that the patient's type of insurance would have a strong impact on all parameters, unfortunately no statistical analysis could be performed due to the small group who had other insurance than Jamkesmas. The presented results do emphasize the low financial resources of this patient group and the need for improvement of the national health care system.

The long waiting time, the neo-adjuvant chemotherapy to overcome the waiting time and the treatment of radiation (with or without concurrent chemotherapy) has impact on patient's physical status. In this study three patients died before treatment could start, one patient died during neo-adjuvant treatment and another four patients died directly after treatment, accordingly 19% died soon after diagnosis. This percentage might be an

underestimation, since directly after diagnosis 11 patients were lost to follow-up. These patients did not get treatment, so it is assumable that some of them also would have died. The results are comparable to a recent study of adults with NPC, conducted in Yogyakarta, here 13% of the patients died before radiotherapy started and 29% died before treatment response could be assessed[2]. Studies involving preservation or improving the patient's physical performance status during the waiting time and during treatment might be of great value to lower the mortality. Suggestions are other treatment modalities, like photodynamic therapy to overcome the waiting time, or protocols to observe and improve the nutritional status[28].

The overall treatment time of radiotherapy was 55 days. Optimally, a total dose of 66 to 70 Gray should be given in 33-35 fractions in a maximum of 47 days. For each day by which radiotherapy treatment is extended, effective dose is lost, and the success rate declines rapidly[29-30]. The long overall treatment time is therefore most probably also a reason for the poor complete response percentage.

Another problem that we encountered was the lack of data management and poor follow-up. This made it impossible to compare the different treatment protocols and made statistical analysis difficult. In general the lack of proper data management causes a lack of essential feedback for doctors, which results in the absence of a learning curve and current insight in problems in cancer care in general. Besides, poor follow-up results in late recognition of recurrent disease, which immediately affects the patient's health and chances of survival. A digital data management system may result in better insights in clinical performance and stimulate the treatment learning curve[31].

CONCLUSION

This is the first study presenting the treatment results of young patients with NPC in Indonesia, where 20% of the patients are diagnosed before the age of 31. Comparable, poor treatment outcome has been found in an independent study among adults with NPC in Yogyakarta, and it is assumable that other low and middle-income countries are coping with similar problems in handling NPC patients[2,28]. The study revealed serious weaknesses at different levels in diagnosis and treatment. The current changes in the insurance system of Indonesia, aiming to provide health care for every one, will put even more pressure on the health care facilities. Therefore it is likely that the problems might get bigger.

Establishing more radiotherapy facilities would be the best step to solve a big part of the problems. However, even when financial resources are not the limiting factor, it will take a decade to built new bunkers and educate doctors and nurses to accomplish this. In the

meanwhile the focus should be to treat people who can have treatment in a proper way. Earlier diagnosis, better treatment compliance and improved follow-up are the key points to accomplish this. More public, medical and patient awareness for these key points might be one of the answers.

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Chapter 7

Temoporfin mediated photodynamic therapy in patients with local persistent and recurrent Nasopharyngeal carcinoma after curative radiotherapy: a feasibility study

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ABSTRACT

Background: The treatment of persistent and recurrent Nasopharyngeal Carcinoma (NPC) remains a challenge, especially in Indonesia. We investigated the safety and efficacy of temoporfin mediated photodynamic therapy (PDT) for patients with local persistent and recurrent NPC.

Material and Methods: Twenty two patients with persistent and recurrent NPC (maximum tumor depth <10 mm) underwent PDT under local anesthesia with use of a nasopharyngeal light applicator. Three different drug doses and light intervals have been administered: treatment arm A: 0.15 mg/kg Foscan®; 96 hours drug-light interval; B: drug dose of 0.10 mg/, 48 hours drug-light interval; C: drug dose of 0.075 mg/kg, 24 hours drug-light interval. Toxicity was measured by using the CTCAE 3.1 scale.

Results: Arm A consisted of eight patients, arm B and C consisted of seven patients. The treatment procedure was well tolerable under local anesthesia. The most common grade III toxicities for all groups is headache (n=7; 33%). No grade IV toxicity was seen. One patient died 2 days after treatment due to a misdiagnosed pneumonia. In 17 of the 22 patients a biopsy was performed after 40 weeks and showed no tumor in all biopsies. Arm A seems, in addition to comparable toxicity, clinically more effective than arms B and C.

Conclusion: The present study demonstrated that temoporfin mediated photodynamic therapy is a relatively simple technique that can be utilized to treat residual or recurrent nasopharyngeal cancer, restricted locally to the nasopharynx.

INTRODUCTION

Nasopharyngeal Cancer (NPC) is endemic in Southern China and most of South-East Asia with a yearly incidence reaching as high as 20-50 cases per 100.000 annually[1,2]. In Indonesia NPC is the most frequent cancer in the head and neck area and is the fourth most common tumor occurring in males. The incidence is estimated 6 per 100,000, leading to at least 14,000 new cases per year[3]. Unfortunately in Indonesia the majority of patients have advanced stage disease at initial diagnosis. In a recent study, 87% of Indonesian patients had stage III-IV disease at initial diagnosis and 18% presented with distant metastasis[4].

Radiotherapy alone or combined with chemotherapy is the treatment of choice for NPC and has a relatively high cure rate[5,6]. In 1999, Lin and Jan reported a failure rate for the primary tumor in patients with T3-4N0M0 NPC of 25%[5]. In 2000, Chang et al. reported a primary tumor control rate of 50-60% after radiotherapy in patients with advanced NPC[7]. During the last decade the treatment results have significantly improved; with disease-free and overall survival results of around 70% and 80%, respectively[8,9]. These improvements are due to the advances in diagnostic imaging, increased radiation dose[10], the different regimes of fractionation, the use of IMRT[11,12], and the use of concomitant chemotherapy[6]. These success percentages cannot be achieved in developing countries mainly due to a lack of radiotherapy facilities, imaging facilities, and poor patient compliance. In Indonesia these problems cause a high percentage of local persistent or recurrent tumor after radiotherapy[4].

Options for treatment of local recurrent NPC are brachytherapy, external re-irradiation, stereotactic radiosurgery and nasopharyngectomy. These treatment modalities can be used either alone or in combination[13-16]. Despite promising local response rates; re-irradiation causes a high incidence of major late complications, such as brain necrosis, cranial nerve palsies, and catastrophic haemorrhages[17-21]. In Indonesia re-irradiation and surgery for locally recurrent disease are not feasible because of insufficient techniques, personnel, equipment, and lack of allocated re-treatment time due to already long waiting times for treatment of primary tumors. In persistent disease, there is limited role for re-irradiation.

Photodynamic therapy (PDT) using a specially designed nasopharyngeal applicator may act as an alternative treatment option since it can be performed easily and does not require expensive equipment and extensive training of physicians and the medical staff.

PDT is a method that involves injection of a systemic photosensitizing drug that can be activated at the tumor site to produce reactive oxygen species, starting a cascade of oxidization of biomolecules and eventually causing tumor destruction. PDT has already

been shown to be an effective modality for treatment of superficial lesions in previously irradiated sites[22-25].

PDT has been studied in the past to treat locally recurrent/persistent NPC. The results from studies with first generation photosensitizers like hematoporphyrin and without a nasopharyngeal applicator for proper illumination, although quite promising, didn't lead to established protocols for PDT for NPC[26]. Temoporfin mediated Photo Dynamic therapy (PDT) is a registered treatment in Europe for incurable head and neck cancer[22,24]. Temoporfin mediated PDT in combination with a specially designed nasopharyngeal applicator for proper illumination of the nasopharyngeal cavity might be a suitable therapy for local persistent or recurrent NPC, especially in developing countries where advanced radiotherapy options are limited[27].

Yow et al has shown in nasopharyngeal cancer cell lines that the uptake of second generation photosensitizer temoporfin is higher and the photodynamic reactions are more efficient compared with hematoporphyrin[28]. Since there are no data reported in the literature on temoporfin mediated PDT in NPC, we planned a study to investigate the feasibility of three different treatment regiments to be able to select one of these treatment regimes and conduct a phase II trial.

METHODS

Patients

Eligible patients were aged 18 years or older with pathologically proven locally recurrent or residual nasopharyngeal carcinoma (type I, II or III), a discrete tumor ≤10 mm in depth measured by CT imaging (surface illumination with Temoporfin has a effective penetration of 10 mm), which is endoscopically visible and accessible for unrestricted surface illumination using a nasopharyngeal applicator. Other eligibility criteria included a Karnofsky performance status of at least 70%. Local ethics committee approval was obtained for the study, and each patient gave written informed consent. All patients had previously been treated with in onset curative radiotherapy for their primary NPC.

Exclusion criteria were any disease which is caused or exacerbated by light and treatment within the prior 30 days with a light-activated therapy. Patients with distant metastasis and lymph-node involvement were also excluded from the study protocol.

Treatment regimes

Three treatment regimes were investigated to select the most safe and efficacious treatment scheme. Besides the standard dose for Head and neck tumors of 0.15 mg/kg, two lower drug doses were evaluated. The possible loss in effectiveness by reducing the drug dose was compensated by reducing the drug-light interval. The lower drug doses were evaluated to see if these would give less side effects and lower the costs with the same effectiveness. Any grade 4 and 5 adverse event (according to CTC 3.1) was considered as SAE (serious adverse event). In case of two SAE from one dose group these parameters were considered too toxic.

Arm A: Eight patients received the dose level and the drug light interval that are recommended for the treatment of patients with squamous cell carcinoma of the head and neck. These parameters are drug dose, 0.15 mg/kg Foscan®; drug-light interval: 96 hours; light dose: 20 J/cm2).

Arm B: 7 patients were treated at a drug dose of 0.10 mg/kg and a drug-light interval of 48 hours.

Arm C: 7 patients were treated at a drug dose of 0.075 mg/kg and a drug-light interval of 24 hours.

Drug administration

A dose of 0.15 mg, 0.10 mg or 0.075 mg of Foscan® per kilogram of body weight was administered by slow intravenous injection (into a proximal deep vein in at least 6 minutes, according to the producers instructions. Patients remained in a light-restricted room for 24 hours after administration of temoporfin and then made a gradual return, with an increase of 100 Lux per day, to unrestricted indoor light exposure over a period of 2 weeks. Guidelines for light exposure and a lux-meter to monitor light exposure were given to the patients, and treating physicians advised both patients and their families and friends about the importance of complying with a gradual return to normal light exposure. A booklet was also available for the patient to optimize adherence to these guidelines.

Laser Illumination

To ensure homogeneous light administration to the entire nasopharynx, a specially designed nasal light applicator (Rotterdam Nasopharyngeal Light Applicator; type 625, Wacker Chemie, Krommenie, The Netherlands was used)[27] (Figure 1). The inner diameter of the silicon tubing can accommodate two linear light diffusers for light delivery and dosimetry.

After decongestion (R/xylometazoline hydrochloride 1%) and topical anaesthesia, the applicator was introduced into the nasopharynx transorally over 2 guiding tubes (four French). By using a small silicone flange, the applicator remains fixed in a stable position during illumination.

The surface of the tumor was illuminated with 652 nm light, emitted from a 6-watt Applied Optronics diode laser, inserted into one channel catheter[27]. The length of the diffuser is 5 cm, and the diameter of the catheter is 0.4 cm. The light dose administered was 20 J/cm at a fluence rate of 100 mW/cm. The total illumination time is 400 seconds for 2 diffusers.

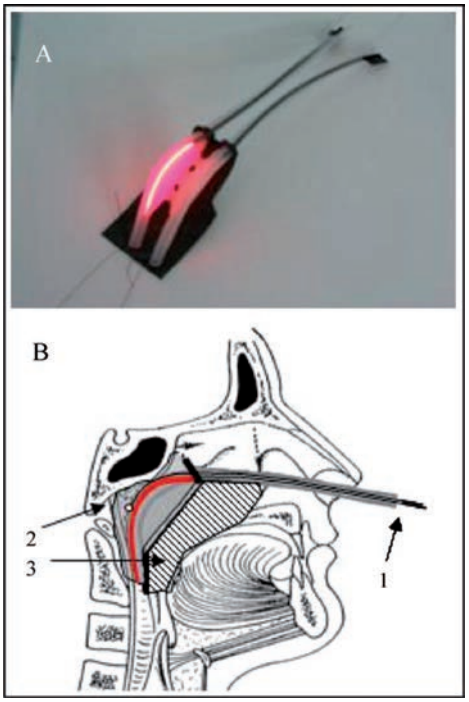

Figure 1 | A: Nasopharynx applicator, B: schematic view of positioning and illumination.
1. Cylindrical diffuser in shielding tube. 2. Target area. 3. Soft palate is shielded. Figure 1 have been used previously by Nyst et al, 2007 and Wildeman. 2010.[27,29]

Study Assessments

Following treatment with PDT, patients were evaluated on day 1 and 2, and week 1, 2, 4, 6, 8, 12 and 16 post PDT. Patients subsequently entered a follow-up phase, with visits every 4 weeks up to 40 weeks post- PDT. Thereafter, they were followed annually until death or lost to follow-up. The following assessments were performed to evaluate safety: physical

examination including vital signs, Karnofsky performance status and weight, recording of adverse events according to CTCAE 3.1, concomitant medication (in particular use of analgesics).

The tumor response was monitored with endoscopic examination of the nasopharynx and biopsies of the nasopharynx. Overall survival was calculated from the time of illumination to death. In case of a partial response or recurrence, based on endoscopic examination and if pathologically proven, the patient was eligible for re-treatment with PDT.

RESULTS

Patient characteristics

Most patients had pathologically proven residual NPC. Only one patient had recurrent disease. Tumor evaluation after the initial treatment, 12 weeks after finishing radiotherapy treatment, showed in the cases with persistent disease tumor in the nasopharynx and were included in this study. The patient with disease recurrence had, during regular follow up, a visible mass at endoscopy which after evaluation showed to be a local recurrence and was included. Tumor size was measured by CT-scan and all patients had a discrete tumor depth of 1 cm or less.

Table 1 | Patient characteristics

	Arm A (n=8) Mean (range)	**Arm B (n=7)**	**Arm C (n=7)**
Age	50 (19-68)	53 (45-61)	41 (43-57)
Male/female	6/2	5/2	2/5
Persistent/ recurrent	8/0	6/1	7/0

Safety/ Adverse Events/ Tolerability

All included patients were able to complete the treatment procedure. The treatment procedure was well tolerable under local anesthesia. There were no short term adverse events. All grade I-IV adverse events scored, until 16 weeks after treatment, are presented in table 2. All possible adverse events were scored but only the post treatment adverse events are presented in table 2.

Three of eight patients in arm A had grade III toxicities for headache; no grade IV toxicity was seen in arm A. One patient in arm A died due to pneumonia 2 days after illumination of the tumor. This is considered a grade V adverse event. A chest X-ray prior to the treatment

already showed pneumonia but this was misinterpreted by the radiologist and despite fever the patient was enrolled in the study.

In Arm B two grade III toxicities were seen in three of seven patients; one patient had a grade III headache, one patient had a grade III tinnitus. There were no grade IV toxicities seen.

Arm C had a total of four grade III toxicities in three of the seven patients. Three patients had a grade III headache; one of these patients already suffered a grade II headache before treatment. One patient had a grade III musculoskeletal myositis of the neck muscles causing a grade III headache. There were no grade IV toxicities seen. One patient Arm C started with a cranial neuropathy grade III that completely resolved one week after treatment.

The most common grade III toxicities for all groups is headache (n=7; 33%). None of the patients had skin burns or other skin adverse reaction caused by the photosensitizer.

Table 2 | Profile of adverse events

Adverse event	Arm A (n=8)*				Arm B (n=7)				Arm C (n=7)			
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4
Mucositis	0	0	0	0	0	0	0	0	1	0	0	0
Xerostomie	2	5	0	0	1	6	0	0	3	3	0	0
Dysphagia	0	7	0	0	3	2	0	0	3	4	0	0
Orbital damage	0	0	0	0	0	0	0	0	1	0	0	0
Headache	2	2	3	0	1	5	1	0	1	2	3	0
Musculoskeletal/ myositis neck	4	0	0	0	1	0	0	0	1	1	1	0
Genealized Muscle Weakness	6	1	0	0	4	3	0	0	2	2	0	0
Otitis external Ear	2	0	0	0	1	1	0	0	1	0	0	0
Tinnitus	0	7	0	0	0	6	1	0	0	5	0	0
Hearing Loss	5	2	0	0	2	0	0	0	1	1	0	0
Ear and labyrinth disorders, effusion of the middle ear	2	1	0	0	3	3	0	0	2	5	0	0
Trismus	0	7	0	0	6	0	0	0	6	0	0	0
Neurophatic pain	5	1	0	0	1	3	0	0	2	3	0	0
Dermatitis	0	0	0	0	0	0	0	0	1	0	0	0
Radiation recall reaction	0	0	0	0	0	0	0	0	1	0	0	0
Rash	0	0	0	0	0	0	0	0	1	0	0	0
Stomatitis/pharyngitis	2	2	0	0	1	0	0	0	1	0	0	0
Total	30	35	3	0	24	28	2	0	28	22	4	0

One patient died due to pneumonia 2 days after illumination of the tumor and is considered a grade 5 adverse event, despite a chest X ray before treatment already showed pneumonia but was misinterpreted by the radiologist

Clinical results

In 17 of the 22 patients a biopsy was performed after 40 weeks and showed no tumor in all 17 biopsies. The patients who didn't receive a biopsy after 40 weeks already died (n=3) or the biopsy could not be performed because the patient didn't show up (n=1) or was postponed (n=1) because of the big earthquake in 2006 and the patient died before the biopsy could be performed[30].

In Arm A one of eight patients died. One patient was lost to follow up but at his last visit, 32 months after treatment, he had no complaints and no signs of disease progression. In Arm B six of seven patients died, three related to disease, one with unknown reason and two not related to the tumor. In Arm C three out of seven patients died, all related to disease and four patients are still alive without any sign of disease.

Survival for all patients is shown in figure 2 and 3. With a mean follow up of 37.8 months (range: 2 days-71 months), 10 patients are alive; two of them had a local recurrence and were successfully re-treated with PDT.

In total 10 patients died, two not related to disease or treatment, one of unknown cause, one treatment related and six related to disease. The 10 patients who are still alive have a mean follow up of 58 months (min 37- max 71 months).

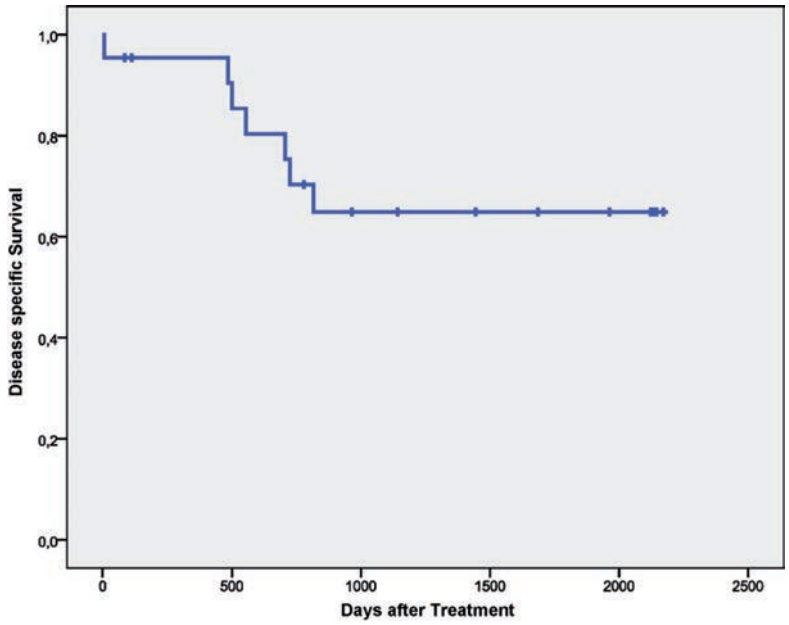

Figure 2 | Disease specific survival

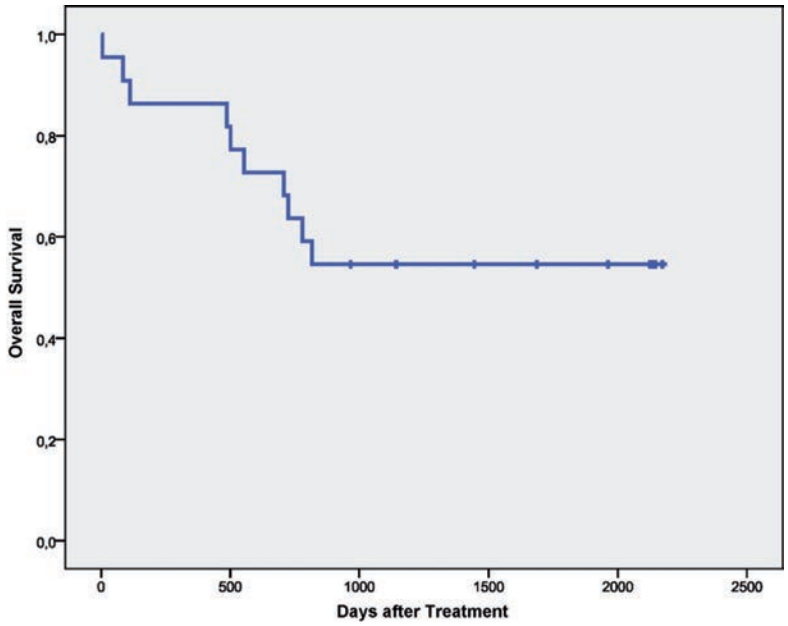

Figure 3 | Overall survival

DISCUSSION

The present study demonstrated that temoporfin mediated photodynamic therapy is a relatively simple technique that can be utilized to treat residual nasopharyngeal cancer, restricted locally to the nasopharynx. All patients tolerated PDT under local anaesthesia in an outpatient clinic setting. Since this procedure takes less than half an hour, it has a significant advantage over other treatment options, which require extensive setups and experienced personnel. The treating physician can administer the PDT alone or with minimal nursing support. Furthermore PDT is a one-time treatment which makes patient compliance to treatment a non-issue, unlike re-radiation where the patient has to show up for multiple radiation sessions. In Indonesia it is frequently observed that patients do not comply with the radiation treatment schedules. Moreover, there is already a long waiting time for radiation treatment even for primary tumors. The same advantage applies over nasopharyngectomy approaches which require a time consuming specified surgical expertise, operating room facilities, time and a longstanding postoperative recovery.

Although this feasibility study is encouraging it is too early to talk about clinical efficacy, which needs to be demonstrated in a larger Phase II trial. Considerable data is available over temoporfin mediated PDT of head and neck cancers[22-25]. However, this was not previously

tested on NPC, which has a very different biological behavior than squamous cell cancers of the head and neck. The undifferentiated histological subtype, NPC WHO III, is the most prevalent NPC type in South-East Asia and Indonesia. This type of cancer is causally associated with the Epstein-Barr virus (EBV)[31]. Therefore we have compared the standard treatment regimen with two other regimens, which are in theory less toxic, to be able to select a regimen for a Phase II trial. Regarding toxicity, there was no significant difference between the treatment groups. The adverse events are mostly local and could be disease related, as well as treatment-related. The most common grade 3 adverse events was headache. The headache seen in the patients can be caused by irritation of the deep neck muscles by PDT, since the prevertebral muscles are close to the nasopharynx and headache symptoms were accompanied by stiff neck feelings. The complaints of headache all disappeared eventually and irreversible damage of the prevetebral muscles is not likely since surface illumination has a penetration depth of 1 cm. The effusion of the middle ear, seen in 16 patients, was caused by massive oedema of the nasopharynx after illumination, which caused occlusion of the Eustachian tube; around 6-8 weeks after illumination the oedema disappeared and the effusion resolved. The death of one patient two days after treatment was probably caused by a missed pneumonia, which the patient already had before the injection of temoprofin.

Arm A seems, in addition to comparable toxicity, clinically more effective than arms B and C. Although clinical complete responses can also be observed in arms B and C, Arm A seems to have a better overall survival and disease specific survival, but the numbers are too small to draw this conclusion. Except for the patient lost to pneumonia, all the patients in arm A are alive and disease free. Arm B and C both have three mortalities due to disease progression. At 40 weeks the biopsies taken from the nasopharynx did not show viable tumor cells. This suggests that all three treatment regimens are effective on the nasopharyngeal surface, but arms B and C might be less penetrating than arm A and missing deeper tumor tissues. The difference in effectiveness could be due to concentration of the photosensitizer. Lower doses of temoprofin might not provide sufficient concentration in the tumor tissues. Because the depth of the tumor receives a lower light fluence than the surface, the amount of activated photosensitizer might not be sufficient to cause cellular damage at certain depths. Whereas with higher concentrations, even though the activation percentage remains the same, the absolute amount of activated photosensitizer could be higher. Another consideration is the location of the photosensitizer in the tissues. Earlier research suggests that the maximal tumor intracellular concentration is reached 4 days after temoporfin injection and in shorter time points, temoporfin is located more in blood vessels[32]. Therefore in arms B and C the treatment effect could be necrosis due to vascular shutdown and in arm A more apoptosis due to intracellular damage. This could explain the better long-term disease control.

Whatever the reason might be, treatment arm A has a longer overall survival and disease-free survival with comparable toxicity/adverse events. Therefore this regimen is chosen to conduct the ongoing phase II study. Once this study is concluded we will be able to determine and compare the clinical efficacy of PDT in patients with small local residual/recurrent NPC.

Based on our experiences so far it is not illogical to assume that Temorfin mediated PDT can also successfully be used in the future as primary treatment modality for superficially growing NPC, with still the availability of all other treatments and PDT for retreatment of small recurrences.

CONCLUSION

PDT could be a very suitable/attractive option for treatment of NPC, in terms of short waiting time, short procedure time which can be conducted under local anesthesia in the outpatient clinic; a simple procedure that can be learned and carried out easily by medical personnel.

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Chapter 8

Short term effect of different teaching methods on Nasopharyngeal carcinoma for general practitioners in Jakarta, Indonesia

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ABSTRACT

Introduction

In Indonesia, Nasopharyngeal Carcinoma (NPC) is the most frequent cancer of the head and neck region. At first presentation in the hospital most patients already have advanced NPC. Our previous study showed that general practitioners (GPs) working in Yogyakarta, Indonesia lack the knowledge necessary for early detection of NPC. By providing training on early symptoms of NPC we hope that the diagnosis and referral will occur at an earlier stage. Here we assess the current NPC knowledge levels of GPs in Jakarta, evaluate improvement after training, compare the effectiveness of two training formats, and estimate the loss of recall over a two week period.

Methods

Two Indonesian GPs visited 31 Primary Health Care Centres (PHCCs) and provided a lecture on NPC. The alternative format consisted of a symposium at the Universitas Indonesia, Jakarta, presented by local head and neck surgeons, with all GPs in the region being invited. To evaluate the effect of both formats a questionnaire was conducted before and after.

Results

The lecture in the PHCCs was attended by 130 GPs. Sixty-six GPs attended the training in the university hospital and 40 GPs attended both. Pre training the NPC knowledge level was poor with an average of 1.6 symptoms being correctly identified out of a potential maximum of 12, this was increased to 4.9 post training ($p < 0.0001$). GPs attending the PHCC course recorded a greater increase in correct symptoms than those attending the symposium (3.8 vs. 2.8; $p=0.01$). After a two week period the knowledge levels had declined slightly from 5.5 correctly identified symptoms to 4.2 ($p=0.25$).

Conclusion

These results confirm our findings regarding GPs insufficient knowledge of NPC. Lectures in the PHCC and a symposium have both been proven to be effective training tools in the education of GPs.

INTRODUCTION

The Indonesian health care sector represents a mix of public and private providers. The government provides primary health care centres (PHCCs). There are more than 7600 of these centres in Indonesia. At the primary health care level, Indonesia has relatively adequate levels of provision, for every 30 000 people one public health centre on average[1,2]. The PHCCs are the first recourse for Indonesians seeking medical attention, with referral to a hospital occurring when deemed necessary.

Cancer is increasingly recognized as leading cause of death in Indonesia, and nasopharyngeal carcinoma (NPC) is the most frequent cancer in the head and neck area and is the fourth most common tumour occurring in males. The incidence is estimated 6 per 100000, leading to at least 14000 new cases per year[3]. However this may be an underestimation due to poor cancer registration. In most countries NPC is an orphan disease with a worldwide incidence of 80000 new cases per year. However, in Southern China and most of South-East Asia NPC is endemic with a yearly incidence reaching as high as 20-50 cases per 100000 annually[4,5].

NPC arises in the epithelial lining of the nasopharynx. This neoplasm is frequently seen at the pharyngeal recess (Rosenmüller's fossa) posteromedial to the medial crura of the eustachian tube opening in the nasopharynx[6].

NPC patients present themselves with symptoms from the following categories: (1) presence of tumour mass in the nasopharynx (epistaxis, nasal obstruction, nasal discharge); (2) dysfunction of the Eustachian tube (tinnitus, hearingloss); (3) skull base erosion and palsy of the 5th and 6th nerve (headache, diplopia, facial pain and numbness); and (4) neck mass (painless enlargement of the upper cervical lymph node). The early symptoms such as epistaxis and tinnitus are not specific for NPC, which makes it difficult to diagnose at an early stage[6].

The Epstein-Barr virus is known as the first tumour virus and was associated with NPC in 1970[7]. Other risk factors of NPC are environmental co-carcinogens, i.e. high levels of volatile nitrosamines and butyrate derivatives in preserved food, especially in salty-preserved fish and dried meat, alcohol and smoking[8-11]. Non-environmental risk factors are gender, ethnicity and family history[12,13].

A majority of patients present with a loco-regional disease most often with an advanced lymph node metastasis in the neck[14,15]. Accordingly the most common symptom at presentation is a painless mass in the neck[15]. Presently, at intake in the hospital Cipto Mangunkusumo hospital/ University of Indonesia, Jakarta 88% of new patients already have advanced NPC. (Adham et al; Chin J Cancer, submitted). The standard treatment

for primary NPC is radiotherapy to which NPC is sensitive, however in advanced cases additional chemotherapy is needed. A recent meta analysis proved the clinical benefit of concurrent chemoradiation therapy compared with radiotherapy alone in the treatment of advanced NPC in endemic areas[16]. The most important prognostic factor is presenting stage[6,17]. Patients with early stage disease (T1, T2 and N0-1 without distant metastasis) can achieve a five years overall survival of 85% compared to 66 % in patients with late stage disease (T3, T4 and N2, N3 without distant metastasis)[17]. The 10-year disease free survival for early stage NPC is 67-71% while for late stage disease this is 29-54%[18].

One possible reason for the high percentage of patients with advanced NPC could be a delay in referral due to poor diagnosis. In our previous study we assessed the knowledge on NPC of the GPs working in the PHCC in the Yogyakarta region[19]. Our results indicated that the knowledge of GPs is insufficient with many not being aware of the high incidence of NPC in their region.

In this study we (1) assess the current knowledge concerning NPC of GPs working in the Jakarta region, (2) evaluate the improvement provided by additional training, (3) compare the effectiveness of two different training formats, and (4) estimate the loss of recall over a two week period. By providing additional training about NPC and its early symptoms we hope to increase the diagnosis and referral of patients with early stage NPC. An early detection program for breast cancer, cervical cancer and NPC was proven to be effective for down staging breast cancer and cervical cancer, however the training was not sufficient to result in a downstaging of NPC[20]. We anticipate our early detection training program to be more effective since we only focus on NPC.

METHODS

Study population

For this study we invited GPs from two of the five districts of the province Jakarta.The study population consists of three groups: (1) GPs who only attended the lecture at their own PHCC; (2) those who only attended the symposium at the hospital Cipto Mangunkusumo (Universitas Indonesia, Jakarta); and (3) those who attended the lecture at the PHCC followed by the symposium at the hospital Cipto Mangunkusumo. Approval for both the visit at the PHCCs as well as for the symposium was given by the head of public health department for the province Jakarta, and a letter of approval was presented at each PHCC visited. For study design see figure 1.

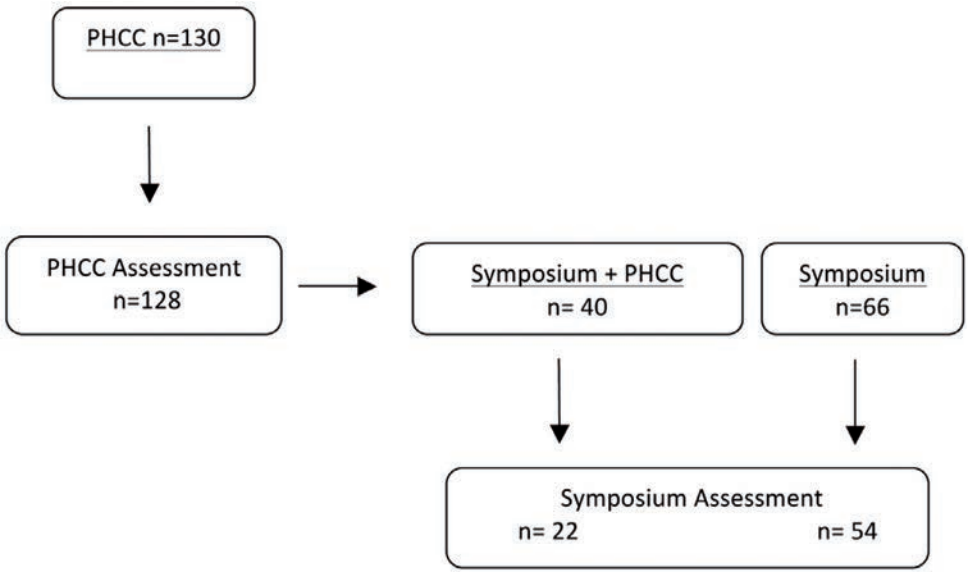

Figure 1 | Outline of the study

Questionnaire

Data were collected utilizing a revised questionnaire, based on the one described in a previous study[19] , which consisted of four sections: (1) general information questions concerning the GP, such as the number of years experience, and the number of patients seen per year; (2) questions concerning NPC, such as early symptoms and risk factors; (3) questions concerning the experience in daily practice regarding the extent to which a GP was confronted with NPC and his/her response for suspected NPC; and (4) questions regarding the ambition and wishes regarding future education on NPC. The questions regarding early symptoms and risk factors allowed for the GP to list as many as he/she thought appropriate, while the questions concerning prevalence of NPC were multiple choice questions. The questionnaire was completed both prior to and after the NPC training session. All questionnaires were finished within 20 minutes, and were made in anonymity.

Primary health care centres (PHCCs)

PHCCs in the Jakarta region were visited by two physicians. The GPs working at these centres were invited to participate in the study. GPs were not informed forehand as to the purpose of the visit. The training session consisted of a lecture designed by a team of Head and Neck surgeons from Rumah Sakit CiptoMangunkusumo/ University of Indonesia, Jakarta,

Indonesia and the Netherlands Cancer Institute/ Antoni van Leeuwenhoek hospital. This lecture focused on all aspects of NPC, especially on early symptoms and best referral strategy. Two Indonesian physicians who have been trained and examined by the same Head and Neck surgeons presented the lecture. The lecture was given in the Indonesian language. The lecture lasted for 35 minutes and afterwards there was time for discussion.

Symposium

All GPs visited by the two physicians in the PHCCs were invited to a subsequent symposium on NPC (one to four weeks thereafter) at the Cipto Mangunkusumo University Hospital in Jakarta, Indonesia. The symposium consisted of a lecture on the risk factors, symptoms, incidence, referral system concerning NPC patient, in addition a physical examination training of the head and neck was provided. The symposium was accredited by the Indonesian Medical Association. The training and lectures lasted for four hours.

Statistical Methods

Analyzed were the questions concerning NPC symptoms, risk factors and age at presentation. The listed NPC symptoms and risk factors were designated as being correct or incorrect, and in the case of the former being one of 12 possible correct symptoms. As a result the number of correctly listed symptoms was binomial (listed or not listed) for the 12 cases. Similarly for the questions concerning youngest and peak age at presentation the outcome was taken as correct or incorrect. While for the number of incorrectly listed symptoms, the number of correctly listed risk factors and the number of incorrectly listed risk factors, we assume the outcome to be Poisson distributed. These outcomes were modelled using generalized linear mixed effects models (GLMMs), either logistic or Poisson mixed effects models. In all models doctor ID is the random intercept with an unstructured covariance matrix. Fixed effect covariates included teaching format (PHCC vs. symposium), time (pre- or post-training), work experience (0-10, 11-20, 20+ years) and the pair-wise interactions of these. The 12 correct symptoms were divided into four categories (for details see figure 2); this covariate and its interaction with other covariates were also included as fixed effects in the model for correct symptoms. The only covariate missing data was the number of work experience years, which was imputed using the median. In these analyses only data from the doctors attending a training session for the first time was employed.

The symposium data from the doctors who had previously attending the PHCC trainings was used to assess the loss in recall over the 2 week period between PHCC and symposium training sessions. In these analyzes the GLMMs included a four level factor representing the

four assessments for these individuals (PHCC pre-training, PHCC post-training, symposium pre-training, symposium post-training) as a fixed effect. The interest being in the change in knowledge between PHCC post-training session and the symposium pre-training session.

In all models, fixed effects were removed if their significance level was greater than 0.10 in a stepwise backwards procedure using log-likelihood ratio tests. No adjustments for multiple testing were performed. A Wilcox-Mann-Whitney test was performed to assess difference in work-experience years between the two groups. For all tests the level of significance set at 0.05.

RESULTS

In total, training sessions were provided at 31 PHCCs involving 130 GPs. All GPs have voluntary participated. Two GPs could not participate in the assessment process as they were attending patients; the remaining present 128 GPs completed the questionnaire both pre and post training. The average number of GPs who participated at a PHCC was 4.3 (median: 3; range 1-12). In total 106 GPs attended the symposium, of which 76 completed both the pre- and post- training questionnaire. Fifty-four of these had not attended the trainings in the PHCCs, while 22 attended both. The overall study population is presented in figure 1.

The clinicians attending the PHCC session had more years work-experience than those attending the symposium (p=0.007). The median amount of years of work experience of clinicians attending the PHCC session was 9 years, with a minimum of one year and a maximum of thirty-three years. Participants at the symposium ranged in work experience from 0 to 27 years (median 9 years). Of the 182 participating clinicians 35 (19%) had more than 20 years experience. Of these, 29 attended the PHCC sessions, while only 6 attended the symposium session.

Symptoms

The overall gain over both trainings was an increase from an average of 1.6 to 4.9 symptoms correctly identified ($p < 0.0001$). However, in comparison with the PHCC group, the symposium group had a smaller overall improvement (p=0.01). Prior to the training sessions an average of 1.4 symptoms were correctly listed at the PHCCs versus 1.7 at the symposium (p=0.30). After training the average number of correct symptoms listed was 5.3 in the PHCC versus 4.5 at the symposium (Table 1).

Table 1 | The mean number of accurately listed symptoms both pre and post training, by GPs attending the PHCC and the symposium sessions.

	Pre-training		Post-training	
	Symptoms correct	**95% CI**	**Symptoms correct**	**95% CI**
PHCC	1.5	(1.3-1.7)	5.3	(5.0-5.6)
Symposium	1.7	(1.4-2.0)	4.5	(4.1-5.0)

There were no difference between GPs with less work experience (0-10 years) and those with moderate work experience (11-20 years) (p=0.46). However compared with these GPs, GPs with longer work experience (20+years) on average listed more symptoms correctly prior to training (1.9 vs. 1.5; p = 0.04), but gained less from the training sessions (4.3 vs. 5.0; p < 0.0001). In the PHCC pre-training questionnaire 71% (129/182) of the GPs correctly identified neck mass as one of the correct symptoms of NPC, however only a few GPs could describe symptoms from one of the other 3 categories in the pre-training assessment (figure 2). Post training there was an increase of correct symptoms for the non-neck mass categories.

Training decreased the number of incorrect symptoms listed by GPs from 2.0 pre-training to 0.9 post-training (p < 0.0001). As with the correctly identified symptoms, this improvement was higher for GPs attending the PHCC training than those attending the symposium (see Table 2; p<0.0001). However GPs attending the symposium recorded fewer incorrect symptoms pre-training than those attending the PHCC sessions (see Table 2; p=0.002). There was no association between the number of incorrect symptoms and number of years of work experience (p=0.85).

Table 2 | The mean number of incorrect symptoms listed in the pre- and post-training assessments by GPs attending the PHCC and symposium training sessions.

	Pre-training		Post-training	
	Symptoms incorrect	**95% CI**	**Symptoms incorrect**	**95% CI**
PHCC	2.4	(2.1-2.8)	0.5	(0.4-0.6)
Symposium	1.6	(1.2-2.0)	1.2	(0.9-1.6)

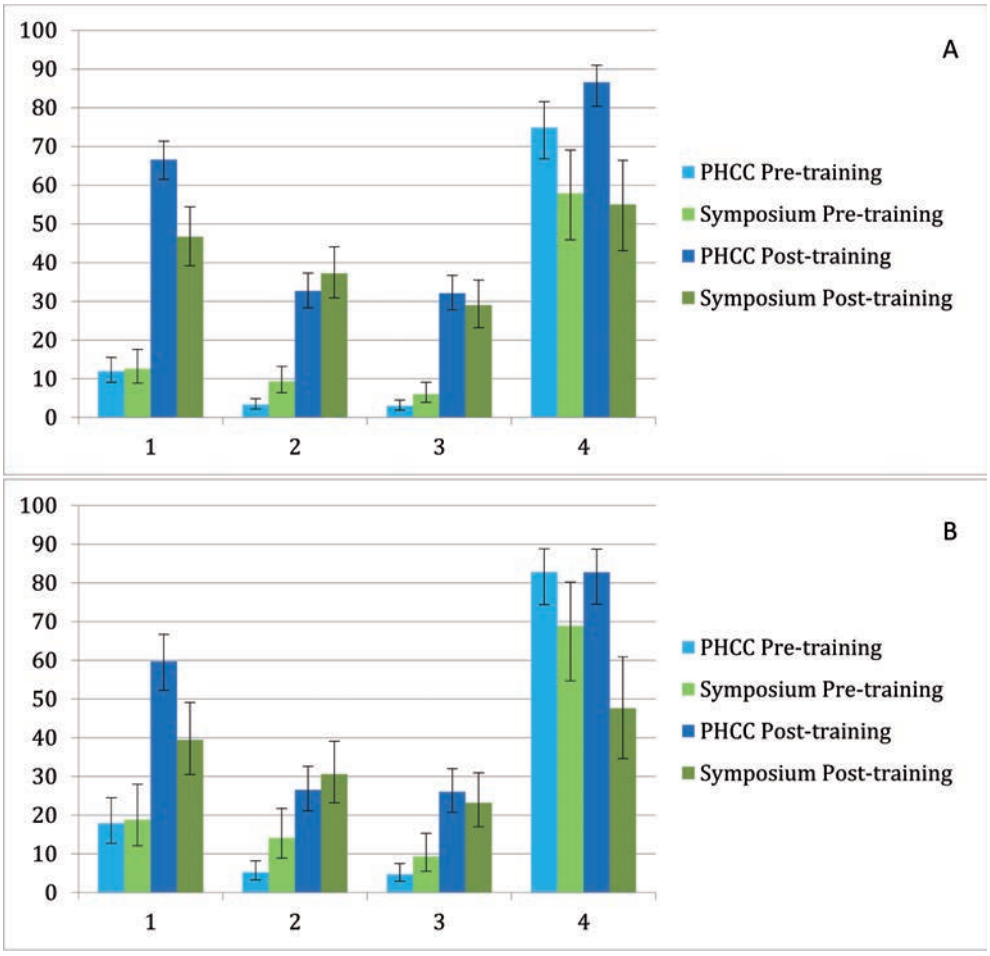

Figure 2 | Percentage of correct answers about the symptoms given by the GPs. a) Percentage of correct symptoms from the four different catagories given by GPs with less than 20 years of work experience. b) Percentage of correct symptoms from the four different catagories given by GPs with more than 20 years of work experience. (1) presence of tumour mass in the nasopharynx; (2) dysfunction of the eustachian tube, associated with the lateroposterior extension of the tumour to the paranasopharyngeal space; (3) skull-base erosion and palsy of the fifth and sixth cranial nerves, associated with the superior extension of the tumour; (4) neck masses.

Risk Factors

The number of correctly listed risk factors increased after the training sessions from 1.5 pre-training to 4.1 post-training ($p < 0.0001$). This improvement was not affected by training format: PHCC vs. symposium ($p=0.76$), nor was the number of years worked related to the number of correct risk factors listed by GPs ($p=0.37$). GPs attending the symposium knew more risk factors prior to training (and therefore after training) than those attending the PHCC only ($p=0.001$) (Table 3).

Table 3 | The mean number of correctly identified risk factors listed in the pre- and post-training by GPs attending the PHCC and symposium sessions.

	Pre-training		Post-training	
	Risk factors correct	95% CI	Risk factors correct	95% CI
PHCC	1.3	(1.1-1.5)	3.8	(3.5-4.1)
Symposium	1.6	(1.4-1.9)	4.7	(4.2-5.3)

Likewise, the number of incorrect risk factors decreased after training (see Table 4; $p = 0.004$); this improvement was not different between those GPs attending the PHCC or symposium sessions ($p=0.16$); there was no association between the number of incorrect risk factors and number of years worked ($p=0.13$); and GPs attending the symposium listed fewer incorrect risk factors pre-training than those attending the PHCC training ($p=0.02$).

Table 4 | The mean number of incorrect risk factors listed in the pre- and post-training assessments by GPs attending the PHCC and the symposium sessions.

	Pre-training		Post-training	
	Risk factors incorrect	95% CI	Risk factors incorrect	95% CI
PHCC	0.8	(0.6-1.0)	0.6	(0.4-0.7)
Symposium	0.5	(0.4-0.7)	0.4	(0.3-0.5)

Age of NPC presentation and peak incidence of age group

The questionnaire also contained questions about the youngest age of presentation and the peak age of incidence. GPs more often answered incorrectly the youngest age at presentation when compared with the peak age at presentation question ($p=0.03$; see Table 5). The training sessions resulted in an improvement in knowledge ($p < 0.0001$), with the PHCC training resulting in a slightly higher improvement than the symposium training ($p = 0.04$). There was no difference between GPs who had worked 0-10 years and those who had

worked 11-20 years (p=0.88), while GPs with the longest working experience (20+ years) more often got the youngest age of presentation question wrong (p=0.03)

Table 5 | The probability (%) of correctly answering questions concerning youngest age of presentation and peak age of incidence.

			Pre-training		Post-training	
		Work Exp.	**% Correct**	**95% CI**	**% Correct**	**95% CI**
Youngest Age	PHCC	0-20 yrs	22	(16-30)	71	(63-78)
		20+ yrs	13	(8-22)	57	(43-71)
	Symposium	0-20 yrs	25	(17-37)	59	(46-70)
		20+ yrs	15	(8-29)	43	(27-61)
Peak Age	PHCC	0-20 yrs	32	(25-40)	81	(74-86)
		20+ yrs	41	(28-55)	86	(77-92)
	Symposium	0-20 yrs	20	(13-31)	52	(40-63)
		20+ yrs	27	(15-45)	61	(43-76)

Assessment of loss of recall: GPs attended who attended both training sessions

GPs who attended both the PHCC and the symposium training sessions had a small reduction in the number of symptoms correctly identified between the end of the PHCC session and the start of the symposium session (5.5 vs. 4.2; p=0.25), however this was still a gain when compared to their pre-training knowledge (4.2 vs. 1.3; $p < 0.0001$). Strikingly, GPs who came back for a second training session recorded twice the number of incorrect symptoms prior to the symposium training (1.1 vs. 0.5; p=0.05), however this was still a large reduction on the number of errors made prior to the PHCC training (1.1 vs. 2.8; $p < 0.0001$). GPs who came back for a second training session recorded slightly fewer correct risk factors pre-symposium training as compared to PHCC post-training (4.1 vs. 3.2; p=0.11), but a significant increase when compared to their knowledge prior to their first training session (1.6 vs. 3.2; p=0.0008). Similarly there was a decrease in the probability of correctly identifying the youngest and peak ages at presentation (Youngest: 84% vs. 59%; Peak: 90% vs. 72%; p=0.39), however GPs still knew more than what they knew prior to the PHCC session (Youngest: 13% vs. 59%; Peak: 22% vs. 72%; $p < 0.0001$).

DISCUSSION

NPC has a high incidence and mortality in Indonesia with late diagnosis being one of the reasons for numerous advanced disease and high mortality. GPs working in a PHCC are the first line of care for patients in need of medical attention. For a correct and early diagnosis of NPC the knowledge of these GPs, especially concerning early-stage symptoms is crucial.

In a prior study in Yogyakarta, Central-Java, we have shown that the knowledge on NPC and related symptoms among GP's working in PHCCs is insufficient for the recognition of NPC and to initiate appropriate referral. Our ongoing studies in the Yogyakarta province and the study from the Jakarta region presented here confirm these results; the GPs working in the Jakarta region have, similar to the Yogyakarta region, insufficient knowledge to refer NPC suspects to the hospital. Besides confirmation of the lack of knowledge, we investigated if improvement of knowledge is possible by introducing a focussed education program. This is the first study that examines the effect of different teaching methods to educate Indonesian GPs on NPC. Early diagnosis will influence the type of treatment patient require; only in advanced stage of disease additional chemotherapy is required, while in early stages radiotherapy alone is sufficient. The addition of chemotherapy to the treatment leads to more serious side effects and a general weakening of the patient. Furthermore, early diagnosis of NPC should lead to fewer patients presenting with distant metastasis who currently cannot be treated with curative intent.

In Malaysia prior studies have proved that the lack of awareness and knowledge of primary health care workers is one of the main reasons for delayed diagnosis. Given that presenting stage is the most important prognostic factor, the appropriate training of GPs is critical[21]. The relevance of adequate referral by GPs for head and neck carcinomas has been showen by Alho et al.[22], who found that in 20% of the 221 patients, subsequently to be diagnosed with head and neck carcinoma, were initially send home without referral. The risk of death in this group was significantly higher when compared with the patients who were immediately referred or received a follow up appointment. Although not statistically significant, patients who were initially sent home had higher cancer stage at diagnosis.

The same research team has also showen that time between GP referral and final diagnosis is a significant factor in patient outcome in other head and neck cancers[23] Long delay in primary care resulted in a significant worsened prognosis especially by patients with laryngeal carcinoma[24].

Educational sessions at PHCCs by a team of two GPs and a symposium by local head and neck surgeons have both shown to be effective. In general, the pre-test knowledge at the symposium was higher than in the PHCC. The reason for this could be that visit in the PHCC was unannounced, while for the symposium the GPs received an invitation so they had some time to prepare for the meeting, or were being pre-informed about the NPC topic by colleagues who had previously attended the PHCC training sessions.

Comparing the two different training formats we see a greater gain in knowledge at the PHCCs. One explanation for this could be that the PHCC sessions provided a more focused

approach and direct contact/confrontation with participants. Another reason could be that the lecture at the symposium was more extensive and perhaps did not delve too deeply into the most important aspects, but rather covered all aspects of NPC to broadly.

On the other hand, during the symposium GPs also received practical education for performing physical examination of the head and neck region and a testimonial of a NPC patient. We expect this additional training aspect to be important for the recognition of NPC patients. Another remarkable result is that the more experienced GPs knew more prior to the training but learned less in both interventions. Perhaps in future the education of longer serving doctors should be adjusted.

For the four different categories of symptoms, as described by Wei et al, we see at the pre-test the most often and only given correct answer is neck mass, which only occurs in advanced NPC. At the post test the GPs are also aware of symptoms in the other categories (see figure 2), most importantly, including symptoms of early stage NPC.

The doctors who attended both trainings showed a small decline in knowledge prior to the symposium when compared with their results directly after the session in the PHCC. However they still knew far more than what they knew prior to the PHCC session. Unfortunately we are only able to assess recall over an average of 8 days. It would be of great interest to assess changes in NPC awareness over a much longer duration.

Visiting the PHCC was a time consuming exercise as the PHCC are scattered throughout Jakarta. The symposium was more time-effective with all the general practitioners present at the same venue and date. Accreditation points are only accrued after the symposium and not the PHCC sessions, thus making the symposium session more attractive for the GPs. However, every GP visited in the PHCC was willing to participate, perhaps stimulated or convinced by the approval letter from the head of public health department of the Jakarta province.

Although this study demonstrates the effectiveness of education GPs about NPC, the time between the pre- and post-training assessments is short. The goal of achieving a down-staging of NPC at presentation is still to be proven. We believe the recent introduction of an online data management service will aid in the confirmation of earlier stage presentation of patients with NPC.[25]

The NPC WHO III histological subtype is the most prevalent type in SE-Asia and Indonesia. This type is causally associated with the Epstein-Barr virus (EBV). Prior studies have shown that EBV-related markers can be used for early detection (screening) and prognostic monitoring. These markers include EBV (IgA) serology and EBV-DNA load since NPC patients

have characteristic elevated IgG and IgA antibody titres to several EBV encoded antigens as well as increased EBV-DNA derived from shed (apoptotic) fragments from the tumour into the circulation. Increased IgA antibody levels are found against early antigen (EA), viral capsid antigen (VCA) and the latent Epstein-Barr nuclear antigen 1 (EBNA1) as well as inhibitory antibodies to the EBV specific DNase[26,27]. These antibody responses against defined viral antigens are the basis of a proposed screening test for NPC in high-risk populations.[28-30] Recent insight in the molecular basis and diversity of anti EBV IgA and IgG responses allowed the development of more defined serological tools[31-35]. A possible assay in the future could be EBV DNA load in the circulation and in nasopharyngeal brushings since both have been detected in a higher proportion of NPC patients than controls[36-40]. Especially EBV IgA serology testing appears to fulfil criteria as a screening tool in the future, since the price is relative low and easy to use when combined with finger-prick blood sampling[34,41]. Future education programs should include referencing to the availability of improved diagnostic procedures for screening and early detection. Improved education combined with a screening method could be a cheap and sensitive screening method for NPC in Indonesia and other high incidence countries. Importantly, the decision for serological or EBV-DNA based analysis has to be based on complaints and duration of complaints registered and interpreted by the GP. Our ongoing research is focussed on finding the best decision tree to accomplish effective early stage diagnosis in NPC high-risk groups (Hutajulu et al; manuscript submitted).

Currently the NPC awareness programme takes place in Jakarta, Yogyakarta and Surabaya. Hopefully in the future this program can be expanded to include all of Indonesia. We also hope to raise public awareness on importance of early-stage cancer and how and when to consult a GP in all layers of Indonesian society. However, as a first step we aim to raise the level of relevant knowledge in primary health care workers. Similar campaigns to educate society on breast and cervical cancer have proven to be highly effective[42,43]. Education of the GPs and society, and combined with improved diagnostic testing will ideally result in earlier detection of NPC, better treatment outcomes, and increased overall prognosis.

CONCLUSION

The current level of knowledge regarding NPC diagnosis is poor, potentially contributing to an increased rate of late stage diagnosis. Additional training sessions increased the knowledge of key symptoms, in particular early-stage symptoms. This increase was observed after conducting both types of training format: a centralized symposium and lectures in local PHCC. With improved knowledge of NPC patients should be referred to hospital at an earlier stage of NPC and as such should have an improved chance of survival.

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Chapter 9

General Discussion

Nasopharyngeal carcinoma (NPC) is a distinct form of head and neck cancer in terms of its etiology, epidemiology, pathology, clinical presentation and response to treatment[1]. In Southern China, the annual incidence is more than 20-50 cases per 100,000[2]. Since there is no appropriate cancer registry in Indonesia, the NPC incidence rate was estimated based on pathology data from the regional hospitals in the year 2000 (11 of 13 pathology centers) that showed an incidence of 5.6/100,000 registered inhabitants[3]. This incidence was similar to the previously described 6.2/100,000 or 12,000 new cases per year[4]. These findings were largely confirmed and extended in our retrospective study as presented in chapter 2[5]. In our reference hospital in Jakarta, NPC was the most frequent head and neck cancer (28.4%) and affected people in the prime of life (peak incidence between 30-50 years of age). Therefore, NPC poses a heavy socio-economic burden on the population. The majority of our patients had an Indonesian origin. This was contrary to our expectations. We expected to have a big proportion of Chinese people due to the high incidence of NPC among Chinese and the high proportion of Chinese people living in Indonesia. Nevertheless, only 10% of the cohort was of Chinese origin. The reason for this might be due to the preference of Chinese people to go abroad for medical care [6,7].

The NPC incidence in Indonesia might be underestimated due to unrecorded cases, since NPC is difficult to recognize and diagnose due to the non-specific symptoms[8] and the lack of awareness for NPC by local general practitioners (GPs)[9]. Improvement of diagnostic tools and more awareness about NPC could contribute to more realistic statistics on the prevalence of NPC.

Most of the patients in our study presented with advanced disease (>90%) and the proportion of distant metastases was high, especially among the young patients[10]. Late stage of disease results in worse prognosis[11–13]. Reasons for the high incidence of late stage might be the high proportion (95%) of cases with undifferentiated carcinomas (WHO type III). They have the highest propensity for lymphatic spread and distant metastases[14–17]. The 5-year survival for stage I disease is 80-90% and 50-70% for stage III-IV[18]. Therefore early diagnosis of NPC is essential for good clinical outcome.

To ensure an early diagnosis, the limited awareness of NPC is problematic. The Indonesian medical student program lacks focus on NPC. This is a shortcoming of their curriculum as well as in the clinical training period[19]. In addition, the GP is replaced by nurses and midwifes during his/her absence from the public primary health care (PPHC)[20,21]. Therefore, education programs on NPC should not only be for GPs, but also for nurses, midwives and medical students. This education is especially important, since most of patients with low socio-economical status will enter via the PPCH[22]. Our study showed that organizing NPC

awareness symposia for the PPCH were effective in improving the knowledge regarding the early symptoms and risk factors for NPC[9]. Therefore they should be continued.

Early diagnosis of NPC depends also on recognition of the clinically non-specific symptoms and the availability of diagnostic assays. Since EBV is present in almost all NPC tissues, the viral biomarkers can be used for diagnosis of NPC. Diagnostic assays based upon EBV immune responses contribute to early diagnosis of NPC. The detection of IgA antibody levels against EBV antigens has shown to be useful in screening for NPC[23–25]. In addition IgA and IgG immune responses against early antigen have been reported to be increased in NPC, related to disease severity[26,27]. The recent changes from original cell-based immunofluorescence tests to standardized and molecular defined ELISA serology has allowed progress to more reliable EBV serology in this field[28,29]. For screening in the field, a simple finger-prick blood sampling can be used to analyze IgA and IgG immune response[30]. However screening would be more efficient when performed in high risk populations, like patients with chronic ENT problems[31]. The presence of aberrant antibody responses as revealed by immunoblot analysis can be used for confirmation[29,32]. Serology can be used as pre-selection or adjunct to traditional clinical diagnostic procedures, but cannot stand alone as a diagnostic marker for NPC[24,33]. A biopsy is needed for accurate confirmation of NPC and remains the gold standard. Tumor cells can be visualized by EBER transcripts by RNA in situ hybridization, to confirm the presence of EBV in NPC[34]. Although biopsies combined with EBER-RISH directly demonstrate the EBV-linked tumor activity, the biopsy sampling is painful and cannot be repeated easily, whereas the testing procedure is very labor intensive. The development of a non-invasive nasopharyngeal brushing technique for obtaining tumor cells from the site of primary tumor origin opens new diagnostic options, as detailed described in Chapter 2 and 3 of this thesis. Brushings can be performed repeatedly, which allows monitoring of treatment responses and follow up of patients. EBV DNA load is used as marker for tumor presence, which can be confirmed by detecting tumor-derived viral carcinoma-specific BARF1 mRNA transcripts in parallel[35]. The level of EBV DNA in brushings was highly discriminative between healthy individuals and patients with NPC. The brushes could be obtained in a reproducible manner and revealed extreme high viral load reaching more than 10 million copies per brush. The nasal-endoscopy guided sampling of tumor cells by brushing was similar to previously used cotton swabs[36–39], but the additional protection of the brush by catheter tubing during nasal passage and novel PCR techniques gave highly improved performance. Measuring viral DNA load in nasopharyngeal brushings could complement simple and cheap EBV based serological assays and allow parallel analysis of the methylation status of host tumor suppressor genes, frequently affected in NPC[28,31]. The combination of these markers would allow early stage NPC detection in populations with defined chronic head and neck complaints and might be useful in risk-assessment in NPC family members, by simple and

affordable techniques, as recently demonstrated[40]. This would allow down staging of NPC at first presentation, thereby improving treatment options and outcome, thus reducing overall morbidity and mortality.

Besides for the initial diagnosis, the viral biomarkers could be used for monitoring the treatment response in patients during follow up. When performed under optimal conditions, the tumor control rate on conventional radiotherapy is 75 to 90% for T1 and T2, and 50 to 75% in T3 and T4 tumors[41], and 90% for N0/N1 cases, and about 70% for N2/N3 cases[42]. However, data from 2007 till 2012 in the Radiotherapy department of Dr. Cipto Mangunkusumo Hospital, the national referral hospital in Jakarta, showed significant lower 3-year and 5-year survival rates for chemo-radiation therapy in NPC patients. Our recent studies from Yogyakarta and Jakarta have shown an actual 3-year survival of less than 30% for adults and 7-19% 5-year survival in young patients[9]. Clinical guidance during and after treatment is needed in order to assess treatment response and early detection of recurrent disease. We studied if diagnostic assays based on EBV could contribute to improve accurate treatment. In 11 patients, repeated biopsies were done to obtain diagnosis. In all 11 patients EBV DNA load in the initial brush was above COV allowing direct diagnosis[10]. More rapid and direct diagnosis will permit timely initiation of treatment, which is of great benefit to enhance NPC treatment success.

The viral load in NP brushings showed steep decrease at 2 months post treatment, which could reflect the response to treatment. The decrease in viral load in local NP brushings as well as in circulating whole blood showed significant correlation with the response to treatment, which is in agreement with Lo et al.[43] suggesting viral load in either compartment might reflect disease activity and may be used to monitor tumor activity. Unfortunately, as was found in an independent previous study in Yogyakarta[44], viral DNA in blood was only detected in a subgroup of NPC patients and mostly at rather low levels compared to parallel NP brushings, irrespective of the tumor size and metastatic activity (TNM). We studied the dynamics of EBV DNA load in blood at defined time points in 68 treated NPC patients. The viral load in blood was not indicative for recurrent disease because most patients kept low positive levels post treatment. Since the viral load in NP brushings is considered to only reflect tumor presence at the primary site[35], evaluation of EBV DNA levels in brush cannot be used to detect distant metastases. In the circulation EBV DNA is fragmented and is probably derived from apoptotic NPC cells releasing their DNA content into the blood[44,45]. Viral load in the blood was detected in 23/68 patients (49%) with NPC in our study, which is similar to the earlier Yogyakarta study mentioned above. After treatment the viral load decreased in most patients to undetectable levels. Previous studies revealed that EBV DNA values close to the COV are not uncommon in peripheral blood[35,46,47]. The reason for the differences

in diagnostic/prognostic value of published EBV DNA levels in plasma/serum (mainly using the BamHI-W repeat fragment for PCR) and whole blood (mainly using single copy gene as target) remain to be resolved, but may be technical in nature. It was recently found that reproducibility of PCR approaches for measuring EBV-DNA in plasma using BamHI-W PCR is rather poor and requires further standardization[48]. Different results have been obtained by Hou et al.[49], showing that pre and post treatment plasma EBV DNA load may have clinical relevance. Pre treatment plasma EBV DNA levels significantly correlated with tumor volume and TNM stage. This would be consistent with the hypothesis that plasma EBV DNA is derived from apoptotic tumor cells in NPC patients and post treatment EBV DNA concentration might be an important predictive factor for distant metastasis[49–51].

The reduction of viral load was not reflected in EBV-IgA serology, which is considered to have a more slow dynamic change in view of the antibody half-life of several months in vivo. However, our study showed IgA antibodies to EBNA1 protein antigen and proved to be a predictor for metastasis. EBNA1 protein plays important roles in the replication and mitotic segregation of EBV episomal genomes[52]. In contrast, IgA antibody levels to the replicative antigen VCA P18 appeared more stable. Overall EBV-IgA serology did not correlate with survival. The major use of EBV-IgA serologic screening is based on steady increases during preclinical tumor development to predict NPC[24].

The expression of the EBV LMP1 gene in NPC is variable and considered to affect tumor behavior[53–56]. We found higher LMP1 expression, observed in NPC patients <30 years of age, which was related to more loco-regional progressivity. In literature, RT combined with multi-agent chemotherapy was effective in achieving a satisfactory Disease Free Survival (DFS) and comparable Overall Survival (OS) in young patients with NPC[57–64]. A study by Sultan et al.[65] revealed better outcome in children and adolescents compared to adults; 5-year NPC-specific survival of 83% ± 3.9% compared to 62% ± 0.8% in adults.

In literature, bimodality in the age distribution is suggested. We did not find a consistent pattern of bimodality as reported by Bray et al., but there was a tendency of more prevalent early age onset, slowly increasing in the young patients/adolescents[61]. The study results on the treatment outcome of young adults were less favorable as compared to literature. In addition, young patients are at higher risk of developing therapy related complications, including second cancer development[65,58]. A promising new approach for NPC treatment in children may be to combine standard therapy with Interferon-beta based immunostimulation as recently reported by Buehrlen et al.[57]. Further studies are needed, to evaluate if NPC in children and adolescents may have different biologic features. Genetic and or environmental determinants which contribute to the development of NPC, maybe different from adult NPC[53,59,62].

The studies in this thesis were hampered by the lack of clinical data management in the hospital, which made it impossible to compare the different treatment protocols and also turned out to be problematic to correlate the EBV-markers to the treatment outcome. Statistical analysis was difficult. However the main and obvious problem was the low treatment success. In Indonesia treatment success is negatively influenced by the low economical state of the health care system. In Indonesia, with a population of 224 million, only 29 radiotherapy centers are present. There are 8 2D devices, 14 3D devices and only 4 IMRT (Intensity Modulated Radiation Therapy) units, which are mostly located in Jakarta region. Patients can only be treated when NPC is proved by pathology and the extension of the disease has been established by imaging. Especially for patients with limited financial resources this is time consuming since they have to ask permission form the insurance company for every diagnostic procedure. In addition, the waiting time for radiotherapy is long. Four months is not exceptional, and currently this delay is getting longer due to the new insurance system, introduced in January 2014, which makes health care available for every Indonesian inhabitant. To overcome the waiting time for (chemo)-radiotherapy, neoadjuvant chemotherapy will be given to the patients, leading to a deterioration of the physical condition. This results in another delay needed for recovery before or during the (chemo-)radiation. Optimal radiotherapy cannot be performed since only 12% of the estimated need is available[66]. Interruption and prolongation of treatment will reduce the benefits of radiotherapy and negatively affect survival[67].

Our study revealed that 84% of the patients younger than 30 years had poor men's insurance (jamkesmas or unwealthy certificate). These patients will have a longer waiting time for radiotherapy as compared to patients with government insurance or private resources. In 2010, an estimated 56 percent of Indonesians mainly state employee, low-income earners and those with private coverage had some form of health insurance. It may increase to 100 percent with a system of universal social health insurance coverage that is started in January 2014. The government's aim is that everybody will have free access to basic/class-3 hospital beds when hospitalization is needed[68,69]. This may be the start of a more equal treatment for everyone. However, more equipment, improving the use of the available units and training of medical staff is mandatory.

Another financial challenge for Indonesian health care is the high outflow of health care dollars to neighboring countries. Many wealthy people choose to get their treatment abroad. Raising medical care to international standards can keep a part of these billions of dollars in Indonesia and be an onset of a more cost-effective health care system.

Other concerns raised in this thesis were the preference of the patients to look for medical help in the alternative circuit. This not only gives a delay to diagnosis, but also causes a

high drop out rate. Even when NPC is diagnosed, some prefer alternative treatment above conventional. For these matters more public awareness is needed. Currently, in Indonesia a program is running to inform the traditional healers that when alternative (cancer) treatment is not effective, they should refer the patients to the hospital.

Local failure is frequently seen in Indonesia. Treatment of local failure is challenging. Surgery and re-irradiation are frequently recommended by NPC-specialized clinics. However, in many hospitals surgical treatment for NPC is not an option due to limited experience with surgery of the nasopharynx and the lack of equipment. Also, re-irradiation is cumbersome, because of the cumulative dose toxicity and the limited radiation capacity. Therefore, readily available and easy to perform alternative modalities are needed. Photodynamic therapy (PDT) has the potential to be a good alternative for treating local NPC[70-73]. It is a minimal invasive procedure using a photosensitizer and laser light. This is a 'one hit' procedure, which can be performed under local anesthesia in the outpatient clinic. In the feasibility trial that we performed for local failures of NPC, PDT was safe, had limited side effects, and was effective in tumor control. Currently, a phase II trial is running in Yogyakarta, to further investigate the efficacy of tumor control of PDT in local failures of NPC. In addition, PDT might be effective in the primary treatment of NPC in Indonesia. The use of PDT could be applied as neo-adjuvant treatment to overcome the waiting time for radiation. Since PDT has limited effect on the physical condition of the patient, in contrast to chemotherapy, postponement or even canceling of concurrent chemo-radiation due to poor condition is unlikely. PDT might be a good alternative to overcome the waiting time, which will be explored in a planned feasibility study.

The treatment results of NPC in Indonesia are not satisfactory; the focus should be on early detection, and improvement of the current treatment. More medical and public awareness on NPC can be the first step. Identification of early stage symptoms is important and EBV markers can contribute to early diagnosis and down-staging NPC. Besides, better follow up is essential to early detect treatment failure, when retreatment is still feasible. The viral biomarkers can provide a diagnostic tool to detect local recurrences and to predict the distant metastasis in the follow up[74]. Furthermore, since EBV is present in all NPC tumor cells, as revealed by EBER-RISH or EBNA1/LMP1 immuno-histochemical staining, the virus itself could be targeted by chemotherapeutic of immunotherapeutic approaches. This is currently under development and evaluated in clinical trials[75–78].

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Chapter 10

Summary/Samenvatting/Intisari

Nasopharyngeal carcinoma (NPC) is highly endemic in Indonesia. It is the most common head and neck cancer and affects patients in the prime of their life, thus posing a big socioeconomic burden on the Indonesian society. Since symptoms of the disease are non-specific and mimic mild flu like complaints, NPC is discovered at a late stage in almost all patients. Knowledge about the NPC incidence and early symptoms among healthcare providers and the population is lacking which contributes to these late referrals. The mainstay of treatment is radiotherapy in early stages combined with chemotherapy in advanced stage. The vast lack of radiotherapy units in the Indonesian archipelago consisting of 17,508 islands totaling 248 million inhabitants makes adequate treatment almost impossible. In total 26 radiotherapy centers with a total of around 8 2D devices, 14 3D and 4 IMRT devices, mostly located in the Jakarta region, are available in the whole of Indonesia. Due to a high number of lost to follow-up (LTF) post treatment, it is difficult to get a clear picture of the real extend of the problems. The high amount of LTF in Indonesia is caused by socioeconomic conditions, long distance to the nearest hospital (archipelago), insurance issues, influence of the whole family in decision making of the treatment and the choice for alternative medicines as treatment. Several studies in this thesis were hampered by LTF.

In this thesis more than 90% of our patients presented at late stage of disease in the clinic, which was reflected in the poor prognosis. The epidemiology and the awareness of NPC were analyzed within the population of Indonesia. Earlier diagnosis would contribute to better outcome, since early stage disease has a better prognosis than advanced stage disease. Since Epstein Barr virus (EBV) is causally associated to NPC and is present in all tumor cells, EBV has the potential as biomarker for diagnostic assays.

The correlation of NPC with EBV opens a new approach for detecting presence of tumor cells by analyzing the viral load in nasopharyngeal (NP) brushings, which eventually might replace the highly invasive biopsy. Quantification of the viral load in NP brushings and blood, in combination with analysis of the humoral IgA immune responses against the VCA-p18 and latent EBNA1 antigen, were used as markers for early NPC detection and monitoring of treatment response.

Chapter 2 describes the epidemiology, incidence, etiology, signs and symptoms at presentation of 1121 patients with NPC treated at dr. Cipto Mangukusumo University hospital in Jakarta in the period of 1996-2005. Indonesia is still an unexplored region with a considerable NPC incidence, averaging at about 6 cases per 100,000 inhabitants. The incidence of NPC at the Dr. Cipto Mangunkusumo University hospital in combination with data from other centers in Indonesia, like, Surabaya, Yogyakarta, Makassar, Bali, Bandung, Medan, shows that the NPC incidence is very high throughout Indonesia, comprising about 12,000 – 15,000 new NPC cases on a yearly basis. Due to the limited cancer registration,

the real number of NPC cases is probably much higher. NPC is the most prevalent head and neck cancer in the Dr. Cipto Mangunkusumo hospital in Jakarta, representing 28.4% of all the cases in the head and neck area. The population with the highest incidence was the Javanese population. However our study showed that almost all ethnic groups in the overall population of Indonesia are affected by NPC. Therefore NPC might not only relate to the commonly thought Chinese genetics, but is a major multi-ethnic problem in Indonesia. Our study revealed that NPC affected patients at a relative young age (peak NPC diagnosis at age of 30-49 years) in Dr. Cipto Mangunkusumo Hospital area. Twenty percent were below 30 years of age. A bimodal age distribution, as reported in other studies, was not seen.

The increasing number of young patients with NPC, may indicate the need for improving awareness and proper screening in the younger patients. The advanced stage of disease at diagnosis, for either the younger as the older patients, might be caused by the nonspecific symptoms at early stage. From all patients, 60.6% were suffering from unilateral ear problems, which belongs to the early symptoms of NPC. Patients indicated that these symptoms appeared already several months before they visited the ENT department.

Since NPC has a high association with EBV, we can use EBV DNA as a tumor biomarker. In **Chapter 3,** a non-invasive brushing technique for obtaining tumor cells from patient was analyzed for its primary diagnostic value. These NP brushing were developed as alternative tests for obtaining tumor cells from the primary site of tumor presentation and to avoid unnecessary painful invasive biopsies in the diagnostic setting. EBV DNA load by non-invasive NP brushing was quantified in patients with NPC and compared to the viral load in healthy individuals and patients with non-NPC head and neck tumors. By using NP brush specimens, we found a significant difference in the level of EBV DNA between NPC patients and control populations. Extreme high EBV-DNA loads (up to 10^7 copies per brush) were found among patients with NPC; while low levels of EBV load (maximally 1.5×10^3 copies per brush) were detected among normal healthy EBV carriers. No correlation was found between EBV DNA load and tumor stage, which might be related to the small surface area reached by the brush. The study showed that accurate EBV DNA quantification using a conserved EBNA1 gene sequence in extracts of nasopharyngeal epithelial cells could reliably detect NPC tumor cells. The sensitivity and specificity of detecting NPC was 90% and 98%, respectively, confirming a previous study that used BamH1 W repeat based DNA amplification. EBV DNA levels in whole blood specimens were not sufficient informative for diagnosing NPC, since EBV DNA levels in many blood samples were negative, despite a positive brush result. Viral load in the NP brush also provided direct information to predict local tumor recurrence post treatment, which is worth pursuing. The viral load in NP brushing procedure reflects carcinoma specific EBV involvement at the anatomical side of tumor development. However

this procedure cannot yet replace biopsy as a diagnostic tool for NPC since histopathology examination from NP area is still considered as the gold standard.

The noninvasive NP brush method as described in the previous chapter was not experienced as painful or compromising for patients and could therefore be used in repeated sampling. In **chapter 4 and 5** the viral load in NP brush and whole blood was described in monitoring of treatment responses during follow-up, in addition to its primary diagnostic use. Definitive diagnosis is made by endoscopic guided biopsy of the primary nasopharyngeal tumor, which is only possible in academic hospitals in Indonesia. However, the insurance system makes a direct referral from the general practitioner to such a hospital impossible. Consequently, an undesired delay in the diagnosis can be caused. In chapter 4 and 5 clinical use of NP brushing versus biopsy, as well as patient experience was investigated. Over 95% of our NPC patients (n=228) revealed high viral load in NP brush samples above cut-off values (COV) determined by local healthy controls (n=53). We demonstrated that EBV DNA load in NP brushings provided a highly specific and minimal invasive tool for primary NPC diagnosis, with similar sensitivity as EBV-IgA serology and superior to EBV DNA levels in blood. Viral load in NP brush samples was not correlated with TNM-status. EBV DNA load in NP brushings significantly declined during treatment, which was not reflected by the humoral immune response against VCA-p18 and EBNA1. Since viral load remained elevated in multiple cases at 2 months post treatment, either the detection of molecular markers alone may not be sufficient as marker for treatment response or this is reflecting poor treatment conditions and outcome. Patients with an EBV DNA load below COV post treatment have better survival at two years. EBV DNA load in blood reflects apoptotic release of DNA fragments, which is rapidly cleared from circulation. High EBV DNA blood levels reflect ongoing tumor apoptosis and necrosis rather than growing tumor mass (chapter 3). Viral load in whole blood obtained 2 months post treatment was in most patients lower than in the diagnostic sample. Since many samples were negative in the follow-up, as well in the diagnostic samples, viral load in whole blood is not a sufficient indicator for treatment responses. The viral load in whole blood did correlate with N and M stages.

The time point of 2 months follow-up chosen for this study may be too short to permit complete disappearance of EBV-DNA loads post treatment. Decreasing tumor related EBV activity is characteristic for complete responders, whereas partial or non-responders have stable or increasing EBV DNA levels. Longer follow-up is needed to proof clinical relevance of persisting EBV DNA level in NP Brush. The follow-up of the patients was extended in chapter 5 to analyze whether dynamics of the viral biomarkers could predict long time responses of treatment and the presence of recurrences. The analysis of viral load after 2 years post treatment showed that the majority of samples were around the clinical COV levels. At the

presence recurrences, viral load in the brush was slightly increased. However, the viral load in blood and the detection of antibodies against VCA-p18 and EBNA1 could not indicate the development of recurrences. The biomarkers as measured at diagnosis did not relate with overall survival time of the patients. However, when the antibody responses after 2 months post treatment remained high, a relation was observed with a better prognosis for the patients (IgA-VCA-p18 p= 0.042 and IgA-EBNA p=0.041). The viral load did not relate with the overall survival time of the patients. The analysis of the dynamics of viral biomarkers was not as indicative for detecting recurrences as expected, but the lack of adequate treatment in combination with irregular sampling made the analysis less reliable.

Chapter 6 describes NPC in children and young adults. Ninety-one percent of these young patients presented at the clinic with advanced disease. There was no difference in features and demography between the children and the young adult group. Of the 49 patients, 14% presented with distant metastasis, while in literature only 1-4% of the children with NPC have distant metastasis at initial diagnosis. This revealed that cancer care of young NPC patients in Jakarta region needs serious attention for down-staging of disease at presentation, appropriate treatment regimens and follow up, in order to increase survival. The low overall survival compared with literature (7-19% versus 52-77%) is caused by the advanced stage at presentation, insufficient treatment and poor socio-economic support

Our study showed that almost all patients had a long waiting time before start of radiotherapy. This represents the already existing pressure on the healthcare system. The ambitious plans of the current government, providing healthcare insurance for the whole population (started from 2014) will increase the pressure on health care even further.

Treatment of persistent and recurrent NPC remains to be a challenge especially in Indonesia, which is described in **Chapter 7**. Residual or recurrent disease in the nasopharynx can be managed with a second course of external radiotherapy. The second dosage should be higher than the initial radiation dose, causing serious side effects. In Indonesia, re-irradiation of recurrent or residual disease is not realistic due to the limited capacity of radiotherapy facilities. Surgical procedures are complicated and need expert skills and adequate equipment. New therapies are needed to deal with this problem. Photodynamic therapy (PDT) is a novel therapy, using a photosensitizer in combination with laser light of a specific wavelength to induce tumor destruction at the illuminated area. We did a feasibility study with 21 patients with local persistent NPC and one patient with local recurrent NPC and treated them with Temoporfin mediated PDT. PDT was safe and effective. PDT with the nasopharyngeal applicator is a relatively simple technique, which can be used to treat residual or recurrent NPC restricted to the nasopharynx. Since this procedure can be performed under local anesthesia and no expensive equipment is needed, the treatment is

a suitable option for regional hospitals in Indonesia. PDT showed to be a “one hit” treatment with limited side effects. In addition to recurrent disease, PDT could be used in the primary treatment setting to overcome the waiting time to radiotherapy that often exceeds the 6 months. A feasibility study is planned to explore this hypothesis.

Since most patients presented in a late stage at the hospitals, efforts must be initiated to provide more knowledge of NPC among healthcare providers and the public. In **Chapter 8** the need for creating awareness about NPC is discussed. A better knowledge of the early symptoms of NPC will result in an earlier referral to the hospitals and as a result down staging of the disease at presentation. General Practitioners (GPs) working in primary health care centers are the first line of care for patients in need of medical attention). For a correct and early diagnosis of NPC the knowledge of these GPs, especially concerning early stage symptoms is crucial. This study indicates that lectures and symposia have proven to be effective training tools in the education of GPs by analyzing questionnaires taken before and after the NPC awareness symposia. Education about early symptoms of NPC, diagnosis and risk factors, resulted in an increased short-term knowledge and suggested need for further training programs. These awareness programs should not only be continued for NPC but should be extended for other types of cancer, since cancer is increasingly recognized as leading cause of death in Indonesia. However a field of tension between the need for early detection and the limited treatment possibilities at this moment gives rise to ethical concerns.

Het nasopharynxcarcinoom (NPC) heeft een hoge incidentie in Indonesië. Het is hier de meest voorkomende tumor in het hoofd-halsgebied. De piekincidentie ligt tussen de 40 en 50 jaar. Hierdoor beïnvloedt het patiënten in de bloei van hun leven en vormt NPC een sociaal-economisch probleem voor de Indonesische samenleving. NPC wordt vaak pas in een laat stadium ontdekt, omdat de eerste klachten aspecifiek zijn en lijken op een verkoudheid en griepklachten. Daarnaast zijn huisartsen vaak niet op de hoogte van de hoge incidentie van NPC in hun regio en missen zij kennis over de symptomen passend bij NPC. Ook de bevolking weet weinig over NPC waardoor zij pas laat naar een dokter gaan bij klachten.

De belangrijkste pijler in de behandeling van een vroeg stadium NPC is radiotherapie. Wanneer de tumor verder gevorderd is, wordt chemotherapie in combinatie met radiotherapie gegeven. Het probleem is echter dat er in Indonesië is een enorm gebrek aan radiotherapeutische faciliteiten. De Indonesische archipel bestaat uit 17.508 eilanden met in totaal 248 miljoen inwoners. Er zijn in totaal 26 radiotherapeutische centra met samen ongeveer 8 2D, 14 3D-apparaten en 4 IMRT apparaten. Deze zijn voornamelijk gelegen in de regio Jakarta. Ter vergelijking, alleen al in de regio Amsterdam zijn net zoveel faciliteiten.

Veel NPC patiënten in Indonesië zullen nooit gediagnosticeerd worden omdat ze überhaupt niet naar het ziekenhuis komen. Ook tijdens en na de behandeling worden veel patiënten uit het oog verloren omdat ze besluiten niet meer terug te komen. Dit, in combinatie met het ontbreken van een landelijk registratiesysteem, maakt het moeilijk om een duidelijk beeld te krijgen van de daadwerkelijke omvang van de NPC incidentie en de gerelateerde problemen. De hoge uitval voor, tijdens en na de behandeling wordt onder andere veroorzaakt door sociaal-economische omstandigheden, lange afstanden naar het dichtstbijzijnde ziekenhuis, verzekeringskwesties, de invloed van de hele familie bij de besluitvorming van de behandeling en de keuze voor alternatieve geneesmiddelen als behandeling. Verschillende studies in dit proefschrift hebben problemen ondervonden door het grote aantal patiënten dat is uitgevallen.

Negentig procent van de patiënten in dit proefschrift presenteerde zich met een laat stadium van de ziekte in het ziekenhuis. De prognose van de behandeling was hierdoor bij de meeste patiënten matig. Een vroegere diagnose van de tumor zal bijdragen tot een beter behandelingsresultaat.

Het Epstein-Barr virus (EBV) is causaal verbonden met NPC en is actief aanwezig in alle tumorcellen. NPC patiënten ontwikkelen een karakteristieke IgA antistof respons tegen EBV eiwitten. Hierdoor kan EBV als potentiële biomarker voor de NPC diagnostiek worden gebruikt. De virale hoeveelheid DNA in de neuskeelholte (nasopharynx) kan bepaald worden met behulp van een borsteltje dat langs de nasopharynxwand gehaald is. Deze methode

kan mogelijk het invasieve biopt vervangen bij de diagnose NPC. In dit proefschrift werd onderzocht of door middel van kwantificering van de EBV-DNA lading in de borstel uit de nasopharynx en in het volbloed, in combinatie met de humorale IgA immuunrespons tegen VCA-p18 en EBNA1, NPC vroegtijdig opgespoord kon worden. Daarnaast werd gekeken of deze biomarkers kunnen helpen bij de controle van de patiënten na de behandeling. Tevens is onderzocht of fotodynamische therapie een optie kan zijn voor de behandeling van NPC en is gekeken hoe de bekendheid van NPC bij artsen kan worden verbeterd.

Hoofdstuk 2 beschrijft de epidemiologie, incidentie, etiologie, en klachten bij presentatie van 1121 patiënten met NPC, die behandeld werden in het Dr. Cipto Mangunkusumo Universitair Ziekenhuis in Jakarta in de periode van 1996-2005. De incidentie van NPC werd bepaald door interne ziekenhuisgegevens te combineren met de gegevens van andere centra in Indonesië, zoals Surabaya, Yogyakarta, Makassar, Bali, Bandung en Medan. De NPC incidentie was zeer hoog in heel Indonesië. Op jaarbasis zijn er ongeveer 12.000-15.000 nieuwe NPC gevallen, neerkomend op een incidentie van 6/100.000. Door de beperkte registratie van oncologische patiënten is het werkelijke aantal NPC gevallen waarschijnlijk veel hoger. NPC is de meest voorkomende tumor in het hoofd-halsgebied (28,4%). De bevolkingsgroep met het hoogste incidentie was de Javaanse bevolking, maar NPC kwam bij alle etnische groepen in Indonesië voor. Aanvankelijk werd gedacht dat met name een Chinese genetische achtergrond een risico vormde voor NPC, maar onze resultaten laten zien dat NPC een multi-etnisch probleem is in Indonesië. Onze studies lieten zien dat patiënten op een relatief jonge leeftijd werden getroffen door NPC (de piekleeftijd was 30-49 jaar). Twintig procent was jonger dan 30 jaar. Een bimodale leeftijdsverdeling, zoals in de literatuur gesuggereerd, werd niet gezien.

Het hoge aantal jonge patiënten met een vergevorderd stadium van NPC geeft het belang aan van vroegtijdige diagnose om betere behandelresultaten te verkrijgen. Van alle patiënten had 60,6% eenzijdige oorproblemen. Deze behoren tot de vroege symptomen van NPC. Patiënten gaven aan dat zij deze symptomen al enkele maanden hadden voordat ze een bezoek aan de KNO-afdeling brachten. Een beter bewustzijn van NPC bij zowel patiënten als dokters en een goede screening van de jonge patiënten met klachten passend bij NPC kunnen bijdragen tot een eerdere diagnose en dus betere behandelresultaten.

De sterke associatie tussen EBV en NPC levert potentiële biomarkers op voor NPC. In **hoofdstuk 3** wordt een niet-invasieve techniek geanalyseerd, waarbij een borsteltje via de neus langs het nasopharynxoppervlak wordt gehaald. De virale DNA lading in de borstel werd kwantitatief gemeten en getest op de diagnostische waarde bij nieuwe NPC patiënten. Deze borsteltjes werden ontwikkeld als alternatieve test voor het verkrijgen van tumorcellen uit de primaire tumor, ter vervanging van de pijnlijke invasieve biopsie. De EBV DNA lading werd

gekwantificeerd en vergeleken met gezonde individuen en patiënten met andere hoofd-halstumoren. We vonden een significant verschil in het niveau van EBV DNA tussen NPC patiënten en de controlepopulaties. Extreem hoge EBV-DNA waarden (tot 10^7 kopieën per borstel) werden gevonden bij patiënten met NPC, terwijl de gezonde EBV dragers maximaal 1,5 x 10^3 kopieën per borstel hadden. Tevens werd gevonden dat EBV-RNA een marker kan zijn voor aanwezigheid van intacte "levende" tumorcellen. Er werd geen correlatie gevonden tussen de EBV-DNA lading en het tumorstadium. Dit kan worden verklaard door het kleine oppervlakte dat bereikt wordt door de borstel. De studie toonde aan dat EBV-DNA kwantificering, in combinatie met de IgA EBNA1, een betrouwbaar diagnosticum is om aanwezigheid van NPC tumorcellen te detecteren.

EBV-DNA spiegels in volbloedmonsters waren niet voldoende informatief voor de diagnose van NPC. Dit kwam met name door het hoge aantal fout-negatieve uitslagen bij NPC patiënten. Na de behandeling zou de borstel ook informatie kunnen verschaffen over de aan- of afwezigheid van lokale tumor. Hiermee zouden recidieven of residuen vroegtijdig opgespoord kunnen worden. De virale lading in de borstels weerspiegelt carcinoomspecifieke EBV betrokkenheid. Nochtans kan deze procedure het biopt nog niet vervangen, omdat histopathologisch onderzoek nog steeds beschouwd wordt als de gouden standaard, maar mogelijk kan dit wel in de toekomst.

In **hoofdstuk 4 en 5** wordt gekeken of de biomarkers, naast de primaire diagnostiek, ook gebruikt kunnen worden om het effect van de behandeling vast te stellen en of ze effectief zijn in de follow-up na behandeling. Tevens werd naar de ervaring van patiënten gevraagd. De afname van de borstel werd niet als pijnlijk ervaren. Definitieve diagnose van NPC wordt gesteld door een endoscopisch geleide biopsie van de primaire tumor in de nasopharynx. In Indonesië is dit alleen mogelijk in de academische ziekenhuizen. Het huidige verzekeringssysteem maakt een directe verwijzing van de huisarts naar een academisch ziekenhuis onmogelijk. Derhalve kan een ongewenste vertraging in de diagnose worden veroorzaakt. Meer dan 95% van onze NPC patiënten (n = 228) bleek een hoge virale lading te hebben in de borstel. De cut-off-waarden (COV) werden bepaald met behulp van gezonde controles (n = 53). We hebben aangetoond dat EBV-DNA load in de borstels een zeer specifiek en minimaal invasief diagnosticum is voor de primaire NPC diagnose. Het had eenzelfde gevoeligheid als EBV-IgA serologie en was superieur aan de bepaling van de EBV-DNA lading in het bloed. De virale lading in de borstel was niet gecorreleerd aan het TNM-stadium. Na de behandeling nam de EBV-DNA lading aanzienlijk af. Dit zagen we niet in de IgA immuunrespons tegen VCA-p18 en EBNA1. De virale lading bleef hoog in meerdere gevallen na twee maanden na de behandeling hetgeen suggereert dat ofwel de detectie van de markers alléén niet voldoende is voor bepaling van de respons ofwel dat er nog

tumorrest aanwezig is als gevolg van slechte c.q. onvolledige behandeling. Dit laatste is het meest waarschijnlijk gezien de lokale behandelsituatie in Indonesië. Patiënten met een EBV-DNA load onder de COV na de behandeling hadden een betere overleving na twee jaar. EBV-DNA load in het bloed weerspiegelt de afscheiding van DNA-fragmenten uit apoptotisch tumorweefsel, die snel geklaard worden uit de circulatie. Een hoge EBV-DNA lading in het bloed zouden aanhoudend tumorapoptose en necrose kunnen weerspiegelen in plaats van een groeiende tumormassa (hoofdstuk 3). Het virale DNA gehalte in volbloed verkregen twee maanden na de behandeling was bij de meeste patiënten lager dan in het initiële diagnostische monster. Aangezien veel volbloedmonsters negatief waren in de diagnostische afnames en in de follow-up, is de virale lading in volbloed geen goede indicator voor de behandelrespons. De virale lading in volbloed correleerde wel met het N en M stadium.

Mogelijk is het gekozen tijdstip van twee maanden na de behandeling te kort om volledige klaring van EBV-DNA te verkrijgen. Afnemende EBV activiteit was kenmerkend voor een complete respons, terwijl gedeeltelijke of non-responders een stabiel of zelfs stijgend EBV DNA niveau hadden. Langere follow-up is nodig om de klinische relevantie van persisterende EBV DNA-lading in de borstel te bepalen.

In **hoofdstuk 5** werd de dynamiek van de virale biomarkers tijdens de follow-up van patiënten uitgebreider geanalyseerd. De analyse van de virale lading twee jaar na de behandeling liet zien dat de meerderheid van de monsters onder de klinische COV lagen. Bij recidiverende ziekte was de virale lading in de borstel licht gestegen. Echter, de virale lading in het bloed en de antilichamen tegen VCA-p18 en EBNA1 waren niet informatief bij de opsporing van recidieven. De biomarkers, gemeten bij diagnose, gaven geen voorspelling op de algemene overlevingstijd van de patiënten. Wanneer de IgA antilichaamrespons twee maanden na de behandeling hoog bleef, werd een betere prognose gezien (IgA - VCA - p18 p = 0,042 en IgA - EBNA p = 0.041). De analyse van de dynamiek van virale biomarkers was niet indicatief voor het detecteren van recidieven zoals verwacht, maar het gebrek aan adequate behandeling in combinatie met de onregelmatige afname van de samples maakt analyse minder betrouwbaar.

Hoofdstuk 6 beschrijft NPC bij kinderen en jongvolwassenen. Negentig procent van deze jonge patiënten in de kliniek presenteerde zich met een gevorderd ziektestadium. Er was geen verschil in demografie en klachtenpatronen tussen de kinderen en de jongvolwassenen groep. Van de 49 patiënten presenteerde 14% zich met afstandsmetastasen. In de literatuur presenteert slechts 1-4% van de kinderen zich met metastasen op afstand. Ook vonden we een zeer lage totale overleving in vergelijking met de literatuur (respectievelijk 7-19% versus 52-77%). Hieruit blijkt dat de oncologische zorg van jonge NPC patiënten in de regio Jakarta serieus aandacht moet krijgen en verbeterd dient te worden. De ziekte zou in een eerder

stadium gediagnosticeerd moeten worden, de juiste behandeling moet op tijd gegeven worden en de follow-up moet verbeterd worden om overlevingskansen te vergroten.

Onze studie toont aan dat bijna alle patiënten een lange wachttijd hadden voor aanvang van de radiotherapie. Dit weerspiegelt de reeds bestaande druk op de gezondheidszorg. De ambitieuze plannen van de huidige regering om een zorgverzekering voor de gehele bevolking te verstrekken (gestart vanaf 2014) zal deze druk verder vergroten.

Behandeling van persisterend en/of recidiverend NPC blijft een uitdaging, vooral in Indonesië. Lokale persisterende of recidiverende ziekte in de nasopharynx kan worden behandeld met herbestraling of chirurgie. Met radiotherapie dient de tweede dosis echter hoger te zijn dan de initiële dosis, waardoor ernstige bijwerkingen kunnen optreden. In Indonesië is her-bestraling sowieso niet realistisch wegens de beperkte capaciteit van de radiotherapiefaciliteiten. Chirurgische procedures voor de nasopharynx zijn ingewikkeld en ook hiervoor ontbreken voldoende voorzieningen. In **hoofdstuk 7** wordt een nieuwe therapie besproken die dit probleem deels kan oplossen. Fotodynamische therapie (PDT) is een therapie waarbij gebruik wordt gemaakt van een lichtgevoelige stof in combinatie met laserlicht om tumordestructie te induceren. We deden een haalbaarheidsstudie met 21 patiënten met lokaal recidiverend of persisterend NPC en behandelden hen met Temoporfine gemedieerd PDT. PDT was veilig en effectief. PDT met de nasopharyngeale applicator is een betrekkelijk eenvoudige techniek, welke kan worden gebruikt om recidieven van NPC, beperkt tot de nasopharynx, te behandelen. Aangezien deze procedure onder plaatselijke verdoving kan worden uitgevoerd en geen dure apparatuur nodig is, is de behandeling een geschikte optie voor regionale ziekenhuizen in Indonesië. PDT is een “one hit” behandeling met beperkte bijwerkingen op de lange termijn. PDT zou ook kunnen worden gebruikt bij de primaire behandeling om de wachttijd voor radiotherapie (vaak meer dan 6 maanden) te overbruggen. Een haalbaarheidsstudie is gepland om deze hypothese te onderzoeken.

Aangezien de meeste patiënten in een laat stadium bij de ziekenhuizen aankomen, moeten inspanningen worden gestart om meer kennis van NPC te verschaffen aan zorgverleners en de bevolking. In **hoofdstuk 8** wordt de noodzaak van het creëren van kennis over NPC onder huisartsen besproken. Betere kennis van de vroege symptomen van NPC kan resulteren in een eerdere verwijzing naar ziekenhuizen, waardoor de ziekte in een eerder stadium vastgesteld kan worden. Voor een juiste en vroegtijdige diagnose van NPC is de kennis van huis- en regioartsen die de eerste zorg verlenen voor patiënten cruciaal. Deze studie gaf aan dat lezingen en symposia effectieve leermiddelen waren om (huis)artsen te trainen. Onderwijs over de vroege symptomen van NPC, diagnose en risicofactoren, resulteerde in een verhoogde kennis op korte termijn. Vragenlijsten lieten de noodzaak van verdere opleiding zien. Deze onderwijsprogramma’s moeten niet alleen worden voortgezet voor

NPC, maar moeten ook worden uitgebreid voor andere vormen van kanker, omdat kanker steeds meer wordt erkend als de belangrijkste doodsoorzaak in Indonesië. Een spanningsveld tussen de noodzaak van betere opsporing en de beperkte behandelingsmogelijkheden op dit moment roept ethische vragen op.

Karsinoma nasofaring (KNF) merupakan kasus endemik di Indonesia dan merupakan keganasan kepala leher yang terbanyak ditemukan. KNF sering terjadi pada usia produktif sehingga menambah beban sosio-ekonomi bagi negara. Gejala KNF seringkali tidak spesifik dan menyerupai keluhan flu, sehingga pada sebagian besar pasien KNF baru diketahui pada stadium lanjut. Pemahaman para petugas kesehatan dan masyarakat awam mengenai insidens dan gejala dini KNF masih sangat rendah sehingga menyebabkan terlambat dirujuk. Terapi pilihan adalah radioterapi pada stadium dini dan kombinasi radioterapi dengan kemoterapi pada stadium lanjut. Masih minimnya fasilitas radioterapi di Indonesia dengan jumlah pulau sebanyak 17,508 dan total penduduk 248 juta jiwa menyebabkan terapi KNF masih jauh dari memadai. Fasilitas radioterapi yang ada sejumlah 26 sentra dengan perangkat 2D sebanyak 8 buah, perangkat 3D sebanyak 14 buah dan perangkat IMRT 4 buah, yang sebagian besar berlokasi di sekitar Jakarta dan melayani penderita KNF dari seluruh Indonesia. Akibat tingginya angka *loss to follow* up pasca terapi, sangat sulit untuk mendapatkan gambaran yang jelas tentang problem secara menyeluruh. Tingginya angka *loss to follow* up di Indonesia disebabkan oleh kondisi sosio-ekonomi, jauhnya jarak ke rumah sakit terdekat (daerah kepulauan), permasalahan asuransi, terlibatnya keluarga besar dalam pengambilan keputusan pengobatan, dan banyak yang beralih ke pengobatan alternatif. Beberapa penelitian di dalam tesis ini sangat dipengaruhi oleh *loss to follow up.*

Pada tesis ini lebih dari 90 persen pasien datang pada stadium lanjut, sehingga prognosisnya buruk. Dilakukan analisis epidemiologi dan pengetahuan tentang KNF pada populasi masyarakat Indonesia. Diagnosis dini akan memberikan hasil yang lebih baik, karena penyakit stadium dini mempunyai prognosis yang lebih baik dibanding penyakit stadium lanjut. Epstein-Barr virus (EBV) diketahui berhubungan dengan KNF dan terdapat pada hampir semua sel tumor. Dengan demikian EBV merupakan *biomarker* yang potensial untuk perangkat diagnostik.

Hubungan antara KNF dan EBV membuka peluang pendekatan baru untuk deteksi adanya sel tumor dengan menganalisis kandungan virus di dalam sikat nasofaring. Cara ini diharapkan bisa menggantikan biopsi yang invasif. Pengukuran kandungan virus di sikat nasofaring dan dalam darah, dikombinasikan dengan analisis respon imun humoral IgA terhadap VCA-p18 dan antigen laten EBNA1, digunakan sebagai penanda untuk deteksi dini KNF dan monitor respon terapi.

Bab 2 menjelaskan tentang epidemiologi, insidens, etiologi, gejala dan tanda saat diagnosis ditegakkan pada 1121 pasien KNF yang mendapat terapi di RSCM periode 1996-2005, RSCM merupakan rumah sakit pendidikan di Jakarta. Indonesia masih belum memiliki data mengenai insidens penyakit KNF, diperkirakan kurang lebih sekitar 6 kasus per 100.000 populasi. Insidens KNF di RSCM digabung dengan data dari sentra lain di Indonesia yaitu

Surabaya, Yogyakarta, Makassar, Bali, Bandung, Medan menunjukkan insidens KNF sangat tinggi di seluruh Indonesia, berkisar 12,000-15,000 kasus KNF baru pertahun. Akibat terbatasnya pendataan kasus kanker, jumlah sesungguhnya kasus KNF kemungkinan lebih tinggi. KNF merupakan kanker kepala leher yang terbanyak di RSCM Jakarta, berkisar 28,4% dari semua kasus di daerah kepala leher. Populasi Jawa merupakan insidens tertinggi. Selain itu penelitian kami menunjukkan hampir semua grup etnik pada populasi Indonesia secara menyeluruh terkena KNF, sehingga KNF tidak saja berhubungan dengan genetik Cina, tetapi lebih merupakan masalah multi etnik yang besar di Indonesia. Penelitian kami mendapati KNF mengenai pasien dengan usia lebih muda (terbanyak pada usia 30-49 thn). Dua puluh persen berusia kurang dari 30 tahun. Distribusi usia *bimodal* seperti dilaporkan pada penelitian lain tidak didapatkan pada penelitian ini.

Meningkatnya jumlah pasien usia muda dengan KNF, menunjukkan perlunya peningkatan kewaspadaan dan skrining yang tepat. Stadium lanjut penyakit pada saat diagnosis, baik pada pasien usia muda maupun lanjut terutama disebabkan karena gejala yang tidak khas pada stadium dini. Dari semua pasien, 60,6% menderita gangguan pendengaran unilateral, yang merupakan gejala awal KNF. Pasien menyatakankan bahwa gejala ini timbul beberapa bulan sebelum berobat ke THT.

Adanya asosiasi yang tinggi KNF dengan EBV, membuat DNA dari EBV dapat digunakan sebagai *biomarker* tumor. Pada bab 3, dilakukan teknik sikat non-invasif sebagai diagnostik primer untuk mendapatkan sel tumor dari pasien. Sikatan nasofaring dikembangkan sebagai tes alternatif untuk mendapatkan sel tumor dari tumor primer dan menghindari rasa nyeri akibat tindakan biopsi pada saat tindakan diagnostik. Kandungan DNA EBV dari sikatan nasofaring yang tidak invasif diukur pada pasien KNF dibandingkan dengan individu sehat dan tumor kepala leher non KNF. Dengan menggunakan jaringan sikatan nasofaring, kami menemukan perbedaan yang signifikan pada tingkat DNA EBV antara pasien KNF dengan populasi kontrol. Ditemukan nilai kandungan DNA EBV yang sangat tinggi pada pasien KNF ($> 10^7$ salinan per sikat), dan nilai kandungan yang rendah (maksimal $1,5x\ 10^3$ salinan per sikat) di antara karier EBV normal yang sehat. Tidak ditemukan adanya hubungan antara kandungan DNA EBV dan stadium tumor, yang mungkin disebabkan oleh terbatasnya permukaan yang dapat dicapai oleh sikat . Penelitian ini menunjukkan pengukuran DNA EBV yang akurat menggunakan sekuens gen EBNA1 yang lestari dari sel epitel nasofaring mampu mendeteksi sel tumor KNF. Dalam mendeteksi KNF, pemeriksaan ini memberikan sensitivitas 90% dan spesifisitas 98%. Hal ini mengkonfirmasi penelitian terdahulu yang menggunakan *BamH1 W repeat based DNA amplification*. Pengukuran kadar DNA EBV pada darah segar tidak bisa memberikan informasi yang tepat dalam mendiagnosis KNF, karena kadar DNA-EBV pada kebanyakan contoh darah memberikan hasil negatif, meskipun hasil

sikat positif. Kandungan virus pada sikat nasofaring juga memberikan informasi langsung untuk prediksi kemungkinan rekurensi tumor primer pasca terapi, jadi patut ditindak-lanjuti. Adanya kandungan virus pada prosedur sikat nasofaring menggambarkan keterlibatan EBV pada karsinoma di daerah anatomis pertumbuhan tumor. Tindakan ini belum dapat menggantikan biopsi sebagai alat diagnosis KNF karena pemeriksaan histopatologi dari daerah nasofaring merupakan baku emas. Metode sikat nasofaring yang tidak invasif, yang diterangkan pada bab sebelumnya tidak menyakitkan untuk pasien sehingga dapat digunakan untuk pengambilan sampel yang berulang.

Pada bab 4 dan 5 dijelaskan bahwa kandungan virus pada sikat nasofaring dan darah segar, selain berguna sebagai diagnosis awal juga dapat digunakan untuk pemantauan respon terapi selama tindak lanjut, Diagnosis pasti ditegakkan berdasarkan biopsi dengan panduan endoskopi pada tumor nasofaring primer, yang hanya bisa dilakukan di rumah sakit pendidikan di Indonesia. Namun sistim asuransi yang ada tidak memungkinkan adanya rujukan langsung dari dokter umum ke rumah sakit tersebut. Akibatnya, terjadi keterlambatan diagnosis yang tidak diinginkan. Pada bab 4 dan 5 dijelaskan evaluasi manfaat klinis sikat nasofaring dibandingkan biopsi, dan juga pengalaman pasien. Lebih dari 95% pasien KNF (n=228) menunjukkan kandungan virus yang tinggi pada sampel sikat nasofaring yang melebihi nilai potong yang diperoleh dari kontrol sehat (n=53). Hasil ini menunjukkan bahwa kandungan DNA EBV pada sikat nasofaring memberikan nilai spesifik tinggi dan merupakan alat diagnostik primer untuk KNF yang minimal invasif, dengan sensitivitas yang hampir sama dengan serologi IgA EBV dan lebih baik dari pengukuran kadar DNA EBV di darah. Kandungan virus pada sampel sikat nasofaring tidak berhubungan dengan status TNM. Kandungan DNA EBV pada sikat nasofaring menurun secara signifikan selama terapi, hal yang tidak didapat pada pemeriksaan respon imun humoral terhadap VCA-P18 dan EBNA1. Karena kandungan virus tetap tinggi pada beberapa kasus 2 bulan pasca terapi, mungkin deteksi penanda molekuler saja tidak cukup sebagai penanda respon terapi atau ini mencerminkan kondisi terapi dan hasil yang tidak baik. Pasien-pasien dengan kandungan DNA EBV dibawah nilai potong pasca terapi mempunyai kesintasan yang lebih baik setelah 2 tahun. Kandungan DNA EBV darah mencerminkan fragmen DNA yang mengalami apoptosis, yang akan dibersihkan dengan cepat dari sirkulasi. Kadar DNA EBV darah yang tinggi mencerminkan proses apoptosis dan nekrosis tumor yang masih berlangsung dan bukan massa tumor yang membesar (Bab 3). Kandungan virus dalam darah yang diambil 2 bulan pasca terapi pada sebagian besar pasien lebih rendah daripada sampel saat diagnosis. Karena kebanyakan sampel memberikan nilai negatif pada saat tindak lanjut, seperti juga pada sampel diagnostik, maka nilai kandungan virus dalam darah tidak dapat dipakai sebagai indikator untuk menilai respons terapi. Kandungan virus dalam darah berhubungan dengan stadium N dan M.

Pemilihan waktu 2 bulan tindak lanjut pasca terapi pada penelitian ini mungkin terlalu singkat untuk proses hilangnya kandungan DNA EBV pasca terapi secara menyeluruh. Pengecilan tumor berhubungan dengan aktivitas EBV merupakan karakteristik untuk respons lengkap, dimana pasien dengan respons sebagian atau tidak merespons mempunyai kadar DNA EBV yang menetap atau meningkat. Perlu periode tindak lanjut yang lebih lama untuk membuktikan relevansi klinis menetapnya kadar DNA EBV pada sikat nasofaring. Tindak lanjut pasien diperpanjang pada bab 5 untuk menilai apakah dinamik *biomarker* virus dapat memprediksi respon terapi jangka panjang dan adanya kekambuhan. Analisis kandungan virus 2 tahun pasca terapi menunjukkan sebagian besar sampel kadarnya disekitar nilai potong klinis. Saat didapati adanya kekambuhan, kandungan virus pada sikat nasofaring sedikit meningkat. Namun, kandungan virus darah dan deteksi antibodi terhadap VCA-P18 dan EBNA1 tidak dapat menunjukkan terjadinya kekambuhan. *Biomarker* yang diukur saat diagnosis tidak berhubungan dengan harapan hidup menyeluruh dari pasien. Namun, apabila respon antibodi setelah 2 bulan pasca terapi tetap tinggi, dapat disimpulkan berhubungan dengan prognosis yang lebih baik bagi pasien (IgA-VCA-p18 p= 0,042 dan IgA-EBNA p=0,041). Kandungan virus tidak berhubungan dengan lamanya harapan hidup pasien secara menyeluruh. Analisis dinamika *biomarker* virus tidak dapat digunakan untuk deteksi adanya kekambuhan, seperti yang diharapkan. Terapi yang tidak adekuat dan ketidak-teraturan pengambilan sampel membuat analisis kurang dapat diandalkan.

Bab 6 memaparkan KNF pada anak dan dewasa muda. Sembilan puluh satu persen dari pasien usia muda datang dengan stadium lanjut. Tidak ada perbedaan gambaran klinis dan demografi antara anak dan grup dewasa muda. Dari 49 pasien, sebanyak 14% datang dengan metastasis jauh, sedangkan pada literatur hanya 1-4% anak dengan KNF menunjukkan metastasis jauh saat diagnosis. Hal ini menyatakan bahwa penatalaksanaan kanker untuk pasien KNF usia muda di daerah Jakarta memerlukan perhatian serius untuk penentuan stadium saat pertama kali didiagnosis, regimen terapi dan tindak lanjut yang sesuai, untuk mengupayakan peningkatan harapan hidup. Rendahnya angka harapan hidup dibandingkan dengan literatur (7-19% banding 52-77%) disebabkan oleh stadium lanjut saat pertama kali datang, terapi yang kurang memadai dan kurangnya dana pada golongan sosioekonomi rendah.

Penelitian ini menunjukkan hampir sebagian besar pasien mempunyai waktu tunggu cukup lama sebelum dimulainya terapi radiasi. Keadaan ini menggambarkan kurang-memadainya sistem pemeliharaan kesehatan sampai saat ini. Rencana pemerintah untuk memfasilitasi asuransi kesehatan untuk semua penduduk (mulai tahun 2014), akan menambah beban pemerintah dalam sistem kesehatan.

Bab 7 menjabarkan penatalaksanaan KNF yang menetap lokal dan yang mengalami kekambuhan, yang merupakan tantangan berat terutama di Indonesia. Kasus residu ataupun kekambuhan di nasofaring dapat ditatalaksana dengan siklus kedua radioterapi eksterna. Dosis kedua perlu lebih tinggi daripada dosis radiasi pertama, dengan akibat meningkatnya efek samping yang akan terjadi. Di Indonesia, pengulangan radiasi pada kasus kambuh atau tumor sisa kurang realistik karena terbatasnya kapasitas fasilitas radioterapi yang merata diseluruh Indonesia. Prosedur operasi pada nasofaring sulit dan memerlukan keterampilan khusus dan peralatan adekuat. Dibutuhkan terapi lain untuk mengatasi hal ini. Terapi fotodinamik (TFD) merupakan terapi baru, menggunakan *photosensitizer* dikombinasikan dengan sinar laser dengan panjang gelombang yang spesifik untuk merangsang terjadinya destruksi pada tumor di daerah yang mengalami iluminasi. Penelitian kelayakan dilakukan pada 21 pasien dengan kasus KNF yang menetap secara lokal dan satu pasien dengan KNF kambuh lokal. Pada pasien-pasien tersebut dilakukan tatalaksana dengan TFD menggunakan Temoporfin. Terbukti terapi TFD aman dan efektif. TFD menggunakan aplikator nasofaring merupakan teknik yang sederhana, dimana dapat digunakan untuk kasus KNF kambuh dan tumor sisa yang terbatas di daerah nasofaring. Karena prosedur ini bisa dilakukan dengan anestesi lokal dan tidak memerlukan peralatan yang mahal, ini merupakan pilihan yang tepat untuk rumah sakit regional di Indonesia. TFD merupakan terapi "sekali tembak" dengan efek samping yang terbatas. Pada kasus kekambuhan, TFD dapat digunakan sebagai terapi primer untuk mengatasi waktu tunggu radioterapi yang mungkin lebih dari 6 bulan. Penelitian kelayakan sedang direncanakan untuk mengeksplorasi hipotesis ini.

Karena sebagian besar pasien datang ke rumah sakit dengan stadium lanjut, sangat diperlukan usaha untuk peningkatan pengetahuan mengenai KNF pada petugas kesehatan dan masyarakat. Pada bab 8 didiskusikan kebutuhan untuk meningkatkan kesadaran tentang KNF. Pengetahuan yang lebih baik tentang gejala dini dari KNF akan meningkatkan rujukan lebih awal ke rumah sakit dan hasilnya penemuan stadium dini pada saat pertama kali datang. Dokter umum yang bekerja di Pusat Kesehatan Masyarakat merupakan lini pertama bagi pasien yang memerlukan perhatian secara medis. Para dokter umum ini diharapkan dapat membuat diagnosis kasus KNF stadium dini. Penelitian ini menunjukkan bahwa kuliah, simposium dan diskusi merupakan alat pembelajaran yang efektif dalam mendidik dokter umum, dengan cara menganalisis kuesioner sebelum dan sesudah pelatihan kesadaran mengenai KNF. Pembelajaran mengenai gejala awal KNF, diagnosis dan faktor risiko dapat meningkatkan pengetahuan dasar dokter umum dan mengindikasikan perlunya program pelatihan lebih lanjut. Program kesadaran tidak saja diperlukan untuk KNF tetapi perlu diperluas untuk tipe kanker lain, karena angka kematian karena kanker semakin meningkat di Indonesia. Namun, adanya masalah pelik antara kebutuhan untuk mendapatkan deteksi dini, dan di lain pihak keterbatasan fasilitas pengobatan, saat ini menjadi permasalahan etis.

List of Publications

1. **Marlinda A.Yudharto**, Alexander C.Vlantis, Edward W.H.To. Radical Neck Dissection for the Management of Regional Recurrence of Nasopharyngeal Carcinoma. Indones J. Oncology.(vol 15). No 1.2004: 12-17

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9. **Marlinda Adham**, Antonius N. Kurniawan. Arina Ika Muhtadi, Averdi Roezin, Bambang Hermani, Soehartati Gondhowiardjo. Ing bing Tan , Jaap.M. Middeldorp. Nasopharyngeal carcinoma in Indonesia: Epidemiology, incidence, signs and symptoms at presentation. Chin Journal of Cancer. 2012:31(4): 185-96

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11. Epstein-Barr virus DNA load in Nasopharyngeal brushings and whole blood in Nasopharyngeal carcinoma Patients before and after treatment. **Adham M,** Greijer AE, Verkuijlen SA, Juwana H, Fleig S, Rachmadi L, Malik O, Kurniawan A, Roezin A, Gondhowiardjo S, Atmakusumah D, Stevens SJ, Hermani B, Tan IB, Middeldorp JM. Clin Cancer Res. 2013 Mar 14.

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Curriculum Vitae

Marlinda Adham was born in 1963 in Jakarta. She learned tolerance since early age, being a moslem who attended a catholic school, at Santa Theresia Jakarta. That was also the place where she found her interest in basketball, in which she became a member of the national team, and went to competitions in Asia. She lost her mother when she was 3 years old, caused by ascending paralysis Landry. The incident turned out to be her biggest inspiration to become a doctor. In 1983 she started her education in medicine at Faculty of Medicine University of Indonesia, also in Jakarta. In 1990 she received the Doctor of Medicine degree (MD). After working for 2 years in Centro Primary Health Care at Dili East Timor, in 1995 she started otorhinolaryngology/ head and neck surgery residency at University of Indonesia's Otorhinolaryngology Head and Neck Department in Dr. Cipto Mangunkusumo Hospital (RSCM) Jakarta. In 1999, she received the title as otorhinolaryngology specialist with the thesis titled Evaluation of Post Laryngectomy in Laryngeal SCC by Assessing Tumor Associated Tissue Eosinophilia (TATE) in ENT Department, RSCM, Jakarta, Indonesia

She started working as a clinical staff at the same department of University of Indonesia RSCM since 2000. She took Otorhinolaryngology/ Head and Neck Surgery fellowship in Chinese University of Hong Kong in 2002, Prince of Wales Hospital Hong Kong under the supervision of Prof Andrew van Hasselt. She was granted a head and neck surgery fellowship in 2004 funded by Dutch Cancer Society in Antonie van Leeuwenhoek Nederland Cancer Institute Amsterdam after meeting her promoters, Prof. Jaap Middeldorp and Prof. Bing Tan in Yogyakarta. After finishing her fellowship at NKI/AVL she started developing Head and Neck training for ENT specialists in Indonesia together with Prof. Bing Tan, Prof. Bambang Hermani, and also supported by Prof. Eugene Myers from Pittsburg USA, Prof. Alfredo Pontejos from Philippines, Prof. Javier Gaviland from Madrid, Prof. Luke Tan Singapore and other colleagues from ORL Centres in Indonesia.

In 2005, Linda started as a clinical research fellow, and became a PhD student at Free University Medical Center Amsterdam under KWF grant in 2006. She took a sandwich programme, which required her to visit Amsterdam every year for 3 or 6 months. In between, she still managed to work in Oncology field, as a staff of academic teaching hospital, and maintain her private practice at 2 other private hospitals, Medistra and Pantai Indah Kapuk hospital, before moving to Siloam MR Comprehensive Cancer Center Hospital. Since 2009, she became a consultant of Otorhinolaryngology-Head and Neck Oncology, and also a secretary of Head and Neck Oncology study group of Indonesian ORL Head and Neck Society. Linda came to be the chief of this study group in 2013. She was also one of the initiators and the general secretary of Indonesian Oncology Doctor Society (PERDOKLI), along with the other colleagues from ORL-HNS, Surgical Oncology, Radiation oncology, Medical Oncology, Histopathology and other departments involved in the management

of head and Neck Cancer in Indonesia. It all started with the realization that to deal with oncology, all specialists need to cooperate and work together as a multidisciplinary team.

Linda's career and experience enabled her to build the international connection with the other Head and Neck surgeons and institutions abroad, something that kept her excited besides dealing with a series of challenging surgeries and cases. However, Linda is more than just a medical practitioner with numerous achievements. She is a single mother who has been juggling between a career in clinical, research, teaching, and private practice. No matter how busy she is, Linda still strives for balance in her life. During her spare time, she does her morning walk swim and music, and also spends the quality time with her beloved children, Adinda and Jordy.

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