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

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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  $1.5 \times 10^3$  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.