Summary

Nasopharyngeal carcinoma (NPC) is the most prevalent head and neck malignancy in Indonesia, with annual incidence rate of 6.2/100,000 population recorded in the region of Yogyakarta. In the local centre, Dr Sardjito Hospital, the last five year report showed that 86% of NPC patients came with late stage disease due to the unspecific symptoms of early stage. Because NPC treatment outcome is stage dependent, this situation undesirably affects the treatment success, putting early detection of NPC as important goal to achieve optimal treatment and better survival.

On a world-wide basis, NPC represents the biggest tumor burden associated with Epstein-Barr virus (EBV) infection. Its association is most clear for the undifferentiated carcinoma (NPC WHO type III) which represents the vast majority (90%) of NPC cases visiting the Dr. Sardjito Hospital in Yogyakarta. The fact that NPC tumors carry clonal EBV genome episomes strongly support the contention that virus infection and active expression of its latency proteins and non-coding RNA transcripts provides the critical, initiating, oncogenic insult. Furthermore, in peripheral blood of NPC patients aberrant antibody response to EBV proteins and elevated viral DNA levels are detected. Because of this causal association with NPC, prior studies have indicated that EBV-related markers can be used for early detection. However, until recently, no suitable test systems were available for early-stage NPC screening. In recent years EBV-based serologies using IgA-based ELISA and IgG immunobloting as well as EBV DNA viral quantification have been developed by EBV group VU University Medical Center and EBV-NPC team Faculty of Medicine Universitas Gadjah Mada, as well as others, allowing for development of improved NPC diagnostic tools in Indonesia. Those new EBV related tests have good diagnostic value in discriminating NPC patients from the regional healthy population, thus are promising for screening. In this thesis their performance in a real screening program was investigated.

To be cost effective, a screening program should target high risk groups of developing the disease. Multiple seroepidemiology and NPC screening studies have demonstrated that individuals presenting with elevated IgA anti-EBV levels and aberrant EBV-reactive IgG antibody pattern as well as those from family with multiple NPC cases are at risk for developing NPC. Besides both groups, individuals with past history of chronic otological and nasal disease were also thought to be high risk population although there is no available serology data yet supporting this observation. In Indonesia, including the local region of Yogyakarta, NPC screening and seroepidemiology studies have never been carried out. Two studies in this thesis tested EBV-based assays for a screening feasibility to defined target groups. The first group was first-degree relatives of sporadic NPC cases considering that family of multiple NPC cases were not frequently present in the local area. The second target was a selected group of people presenting with chronic complaints
in the head and neck, suspicious of being NPC. In this group early identification of
NPC was also carried out. EBV IgA ELISA was used as the first-line screening test in
both populations based on its diagnostic value, relative low cost and the feasibility
to use a simple finger prick method for serum collection, allowing its application in
a large scale population-based study. As confirmation test for EBV IgA ELISA, the
IgG immunoblot test was used and viral DNA load was measured by quantitative
real-time PCR in selected symptomatic individuals in a smaller scale hospital-based
study.

To further contribute to potential new and improved NPC marker tests, this
thesis also described the development of an antibody test against BamHI-A rightward
frame-1 (BARF1) protein. The strong association of BARF1 gene expression with
EBV-driven epithelial malignancies, including both NPC and 10% of gastric cancers
world-wide, might provide an alternative or complementary marker for population
screening. For this purpose, the frequency of BARF1 mutation in the NPC cases from
Indonesia was analyzed.

Considering the presence of a so-called “serologic window” in individuals
developing NPC, better selection of high-risk persons will allow an earlier detection
rate and minimalize false positives. This thesis also described CpG methylation
analysis of multiple NPC-related tumor suppressor genes (TSG) to see whether
methylation status had potential complementary value to EBV-based parameters.

Chapter 2 addressed the question whether family members of scattered
NPC patients have higher risk of NPC compared to the general community. A total
of 520 first-degree relatives and 86 healthy NPC-matched controls were recruited
in a home-visit study. A comparison of aberrant IgA reaction against EBV antigens
showed that first-degree relatives of NPC patients had similar pattern of antibody
responses compared to the community at large (41.2% versus 39.5%). Selecting
for high level EBV IgA seroreactivity, the NPC family members showed significantly
more frequent elevated EBV IgA than community controls (36.9% versus 14.7%). In
general, the elevated response may reflect more frequent productive EBV replication
and viral reinfection and indicates higher risk for the disease within NPC families
compared to community at large. This population may serve as suitable candidates
for more intensive screening and long monitoring to anticipate early-stage of
malignancy. The high level of EBV IgA seroreactivity in ELISA is an appropriate point
for initiating clinical and serology follow-up. To see the possible influence of shared
environmental exposure exogenous factors were compared among the first-degree
relatives on NPC cases. The analysis showed that none of the environmental factors
in the local region appeared to significantly correlate with the IgA seroprevalence
among family members.

Chapter 3 described an early-stage NPC identification approach using a
3-step protocol of EBV-based assays evaluated among individuals presenting with
chronic complaints in head and neck. The EBV-based parameters consisted of EBV
IgA ELISA as the main marker, the same assay used in chapter 2, subsequently
confirmed with IgG immunoblot and EBV DNA load quantification in non invasive
nasopharyngeal brushings. The study included a total of 217 individuals with various head and neck complaints selected during year 2007-2010 and demonstrated a 34.6% seroprevalence based on EBV IgA serology. After IgG immunoblot and viral load confirmation, further clinical investigation identified three defined NPC cases (1.4%) including two cases of early stage. IgG immunoblot confirmed all NPC cases found and EBV DNA load confirmed the one brushing available from these cases. Another case with EBV positive hyperplasia, considered as precursor lesion to NPC, was also identified. This study is the first to verify individuals with symptoms in head and neck as high risk group for an NPC case finding program. This result suggests that the EBV-based assays may serve as valuable tools for NPC early detection. Periodic follow-up is continued in individuals showing EBV IgA seropositivity at baseline to anticipate other NPC cases. To this date the follow-up of the EBV IgA seropositive individuals has not yet yielded new NPC cases.

BARF1 is a carcinoma-specific EBV-encoded oncogene. BARF1 may be more important for NPC oncogenesis than the EBV LMP1 oncoprotein. The specific BARF1 gene expression in NPC may have value for NPC diagnosis and screening. Analysis of BARF1 DNA sequence variation is important for setting up a new diagnostic approach. **Chapter 4** described the BARF1 DNA sequence diversity in nasopharyngeal brush isolates of NPC in comparison to blood or tissue from other EBV-related diseases and spontaneous B cell lines (LCL) of healthy EBV carriers. Most NPC isolates demonstrated specific BARF1 nucleotide changes compared to prototype B95-8 virus leading to three main amino acid substitutions (V29A, W72G, H130R). These conversions are not considered to significantly affect the gross tertiary structure of the hexameric BARF1 protein. Indonesian NPC isolates showed significantly more frequent codon mutations than Caucasian non NPC samples but insignificantly compared to Indonesian healthy LCLs. The sequence variation of BARF1 observed in Indonesian NPC patients and healthy controls may reflect natural distribution of EBV strains unlikely to be predisposing to carcinogenesis. However, the conserved nature of BARF1 may reflect an important role in EBV (epithelial) persistence.

To analyse potential of antibody against BARF1 as a new diagnostic tool for NPC diagnosis, **chapter 5** gave details on expression and purification of NPC-derived BARF1 comprising the two conserved and most prevalent mutations described in **chapter 4** (the V29A and H130R). This mutant BARF1 protein was expressed and secreted as a hexameric molecule in human HEK293 cells and purified to homogeneity by lectin-affinity chromatography. Purified native hexameric BARF1 protein isolated from culture medium was used in ELISA. Considering that BARF1 mutation might affect immune recognition, the humoral immune responses against prototype (B95-8) BARF1 and purified native hexameric BARF1 were compared in sera NPC patients and healthy EBV-positive and EBV-negative individuals. This study showed relatively weak IgG and IgA responses to BARF1 in human sera, much weaker than antibody responses to other EBV antigens (i.e. IgG against EBNA1 and VCA-p18). However, NPC patients had significantly higher IgG-BARF1 reactivity than healthy controls. These results indicate that BARF1 is not a strong immunogen.
Although the use of native hexameric BARF1 protein proved essential for detecting anti-BARF1 antibody responses in NPC patients, the responses are too low to be used for NPC diagnosis.

Chapter 6 evaluated the methylation status of multiple TSGs in order to define whether this may be of added value to NPC early detection based on EBV parameters. This study tested the methylation status of ten NPC-related genes including DAPK1, CDH13, DLC1, RASSF1A, CADM1, p16, WIF1, CHFR, RIZ1 and RASSF2A. Each TSG contributes to the multistep of NPC oncogenic process, including alterations of apoptosis, cell cycle and mitotic checkpoint regulation, intracellular adhesion, cytoskeleton organization and Wnt-signalling pathway. Methylation-specific PCR (MSP) was performed on nasopharyngeal brushings of 53 NPC patients, 22 high risk subjects and 25 healthy EBV carriers. NPC samples showed methylation for individual TSGs in the range of 29.2%-79.2%, most of them comparable to previous reports. High risk individuals, determined by elevated EBV IgA and viral load, showed higher frequency of methylation of CDH13, DAPK1, DLC1 and CADM1, and low frequency and undetectable methylation of the other six genes. A combination of RASSF1A and p16 gene promoter methylation rendered good discrimination between NPC and non NPC, but best results were combined analysis of five methylation markers (RASSF1A, p16, WIF1, CHFR and RIZ1) with detection rate of 98%. Since methylation status had no significant correlation with EBV IgA seroreactivity and viral DNA quantity, this multiple marker MSP is proposed as a complementary test for NPC risk assessment in combination with EBV-based markers. Furthermore, NPC paraffin and brushing DNA revealed an 81.8% concordance underlining the role of nasopharyngeal brushing sampling as appropriate method for NPC screening. This study also performed a pilot methylation test for MAL gene, which has never been evaluated in NPC, showing this TSG to be another promising marker for NPC.

Chapter 7 exploited the increasing evidence that indicates inactivation of TSGs by aberrant promoter methylation as early event in the pathogenesis of tumor formation. Aiming at developing early diagnostic and prognostic tools for various tumors, we took an EBV-associated tumor, nasopharyngeal carcinoma (NPC), as a model and developed a powerful assay based on multiplex methylation specific-PCR (MMSP). The designed MMSP assay defines the tumor-specific methylation status of defined NPC-related TSGs together with EBV DNA detection and LMP1 oncogene expression, providing multiplex information simultaneously in a single PCR reaction with tiny DNA samples from nasopharyngeal epithelium at the site of primary tumor formation. This study collected paired nasopharyngeal swabs and NPC biopsies from 49 NPC patients and 20 non cancerous controls. The panel of markers in this NPC-specific-MMSP assay includes two EBV genes, EBNA1 and LMP1, and two cellular TSG markers. The working condition of MMSP was optimized so that it provides information equal to that from the corresponding separate PCRs. The results showed that MMSP patterns of NPC swab were largely consistent with those of corresponding biopsies and significantly differed from those of 20 non cancerous
controls. Among the 69 samples (49 NPCs and 20 normal controls), the sensitivity of detecting NPC from nasopharyngeal swabs was 98% with a specificity of 100%. The nasopharyngeal swab-based MMSP assay is a non invasive, highly reproducible and informative method, with high potential as a diagnostic tool for NPC.

In conclusion, the studies in this thesis have built on previous developed EBV-based methods for NPC diagnosis and shown their feasibility for general use in Indonesia. Considering that early-stage recognition of NPC will be socially desired and economically feasible, as further options for NPC downgrading in our local region, a clinical and serological follow-up of EBV IgA seropositive individuals should be explored. EBV-based parameters, especially the relatively simple and economical EBV IgA ELISA may be is clinically useful as first screening marker, which can be performed on either venous blood or finger prick dried-blood. For serological confirmation the IgG immunoblot diversity test may be used. The more costly EBV DNA detection in nasopharyngeal brushings proved a promising complementary marker to the initial EBV serological screening approach. The methylation marker analysis advocated by this study can be used as another complementary test with added value to EBV-based assays. The MMSP assay consisting of two methylation markers together with EBV detection may materialize these markers in a single test for early-stage NPC screening which should be subject for further investigation. Multiple independent markers with complementary value may be the best option for early-stage NPC screening, similar as now propagated for cervical cancer screening using papillomavirus-based DNA PCR test, complemented with methylation markers. However, financial constraints have to be considered in developing countries such as Indonesia. Screening individuals with defined suspicious head and neck symptoms and/or a family history of NPC will have a high compliance to the screening program, because of inherent self-motivation. These groups of high risk people should be monitored regularly such as in every 6-12 months because positive results of EBV-parameters, plus methylation of certain TSGs, suggest a sub-clinical early-stage nasopharyngeal tumor process. The clinical examination should include nasopharyngeal inspection by nasendoscopy with brush sampling to anticipate early-stage malignancy in the future.

In this thesis antibody response to BARF1 was found not very useful for NPC marker. However, the detection of BARF1 as a secreted protein in the circulation is another promising diagnostic option for further exploration. The EBV-encoded BARF1 RNA or BART-derived microRNA in brushings or packaged into exosomes in the circulation may provide further novel NPC markers. Together with the markers described in this thesis these new markers will allow more fine-tuned recognition of early events in the EBV-driven and environmentally enhanced carcinogenic accidents leading to NPC. Additional activities at the clinical, public and political level are needed for improving awareness of early-stage malignancy and current diagnostic options to improve early treatment of NPC and to reduce the burden of this prevalent malignancy in Indonesia.