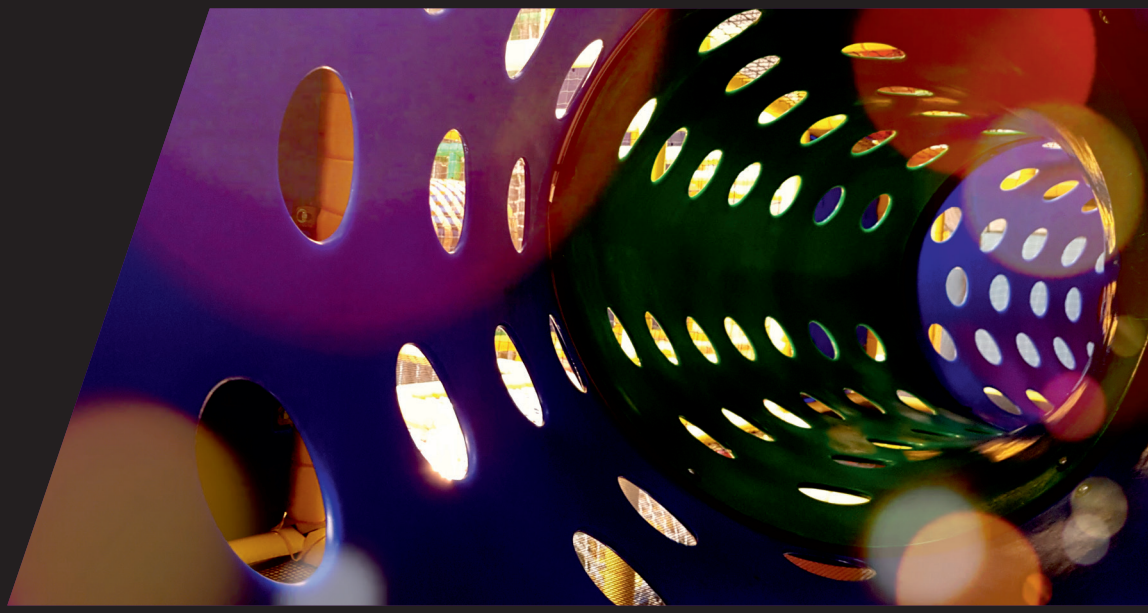
Epstein-Barr virus diagnostic and therapeutic opportunities in nasopharyngeal carcinoma



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"If you want to fly, you have to give up everything
that weighs you down"

Toni Morrison, Song of Solomon

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Introduction and outline of the thesis

Introduction

Pathogenesis of Epstein–Barr virus (EBV) and its contribution to the overall burden of human cancer death

EBV is a DNA virus of the gammaherpesvirus family that infects >90% of the global population[1,2]. Though the infection with EBV is usually benign in the acute stages and latent in the chronic phase in the vast majority of people, the virus has been demonstrated to be associated with a range of human cancers. EBV-associated cancers originating from epithelial cells, lymphocytes and mesenchymal cells include nasopharyngeal and gastric carcinoma, Hodgkin's and non-Hodgkin's lymphomas (especially Burkitt lymphoma), lymphomas and lymphoproliferative disorders in the immunocompromised patients[1-3]. It has been estimated that EBV infection is responsible for about 200,000 new cases of cancer every year – and more than 140,000 deaths – worldwide (Table 1)[1,2,4-6]. One of the major contributors to this total is nasopharyngeal carcinoma (NPC), but the greatest single contribution to the global burden of EBV-associated cancers is estimated to be gastric cancer (EBV-associated gastric cancer called EBVaGC)[4].

Table 1. Estimated new cases of EBV-associated cancers worldwide per year

Cancer	Number of cases	Number of cases attributable to EBV
Burkit lymphoma:		
Sporadic	400	100
Endemic	7,800	6,600
Gastric carcinoma	933,900	84,050
Hodgkin lymphoma	62,400	28,600
Post-transplant lymphoproliferative disease and AIDS-associated non-Hodgkin lymphoma (PTLD & NHL)	1,900	1,520
NPC	80,000	78,100
Total		198,970

Summarized from references 4,6

EBV has two distinct life cycles in the human host; a lytic form of infection that produces new infectious virions, and a latent form of infection that allows the virus to persist in a dormant state for the lifetime of the host[6-8]. Primary EBV infection of adolescents and young adults induces a self-limiting disease, also known as infectious mononucleosis (IM) in about half of the individuals. The virus initially infects epithelial cells in the naso- and oro-pharyngeal mucosa of the upper respiratory tract and they naturally exist in lytic state. B lymphocytes are infected as they traffic in close proximity to these cells. Healthy EBV carriers and IM patients then regularly release transforming virus into the

saliva for transmission[1,2,4,5]. The sequence of events is, however, still controversial[9,10]. Following primary infection, EBV persistently enters the circulating B cell pool of the immune system[1,3,9-12]. Although most individuals with IM recover uneventfully, in some rare cases, more common in East Asian individuals than those of other heritages, infection of adolescents and young adults with EBV can become chronically active, with a high mortality rate[13].

EBV has evolved a life cycle that mimics the natural differentiation pathway of antigen-activated B cells, giving the virus access to its site of latency, the resting memory B cells[12]. By steering infected cells through the various stages of lymphocyte differentiation, EBV is able to establish permanent residence in a cell type that suitable for long-term latent persistence and periodic reactivation[10-12]. EBV latent genes play an essential role in EBV-induced host cell transformation and immune evasion, although lytic EBV reactivation also contributes to the development of EBV-associated neoplasm[5,7,8].

EBV produces microRNAs (miRNAs) which block viral genes that are important for the switch from latent to lytic replication. The activity of viral miRNAs — which themselves lack antigenicity — is believed to contribute to EBV latency allowing lifelong viral persistence in infected cells, despite the presence of a functional host immune system[8]. By enhancing transmission of the virus from cell to cell, EBV lytic infection may increase the total number of latently-infected cells and thus is an essential aspect of viral pathogenesis[7]. This is most clearly demonstrated in acquired immunodeficiency syndrome (AIDS)-associated Oral Hairy Leukoplakia, the only disease that directly linked to EBV lytic replication in epithelial cells[2,14]. Recent data on persistent infection of EBV in B cells have revealed more complex scenario. While establishing latency in memory B cells, the virus might transiently undergo spontaneous lytic reactivation and/or abortive infection in minor subpopulation of such cells[14-16]. To sum up, EBV has evolved efficient strategies to infect B cells and hijack their cellular machinery in order to maintain its persistence and latency[9,10,17].

The contribution of the viral infection to cancer development (Table 2) is best evidenced in patients with a weakened immune system such as transplant recipients and patients with human immunodeficiency virus (HIV)/AIDS. These patients develop lymphomas expressing all EBNA and LMP proteins (latency type III, Table 2), the pathogenesis of which is well-understood[3]. In the majority of cases, it remains unclear how EBV infection leads to cancer development[2,3,4,6]. In the specific setting of Burkitt (BL) and Hodgkin (HL) lymphomas, the risk of a cell carrying a genetic accident such as somatic mutation and then progressing to malignant lymphoma is greatly enhanced when the cell involved is also infected with EBV[4,9,13].

Cellular genetic changes such as uncontrolled activation of a cellular oncogene c-myc triggered by chromosomal translocation, likely results from acquired immunodeficiency (AID)-driven aberrations in immunoglobulin gene rearrangements[4,18]. EBV prevents apoptosis in such "damaged" cells by regulating the overexpression of survivin and Bcl-2 family proteins. Epigenetic silencing of the proapoptotic gene BIM1 by the virus also leads to prolonged cell survival[19-21]. More importantly, in both lymphomas, EBV contributes to tumor cell growth and survival through restricted forms of infection, involving just a subset of latent proteins such as EBNA1, LMP1 and LMP2A (latency type I and type II; Table 2)[2-4]. However, pathogenesis models for EBV-associated lymphomas are not universally accepted. Recent large-scale epigenomic analysis of BL and LCL cells supports the idea of EBV induced-epigenetic alterations following transient infection with the potential to enhance cellular motility and invasion. These stable epigenetic alterations that remain as a footprint of past EBV infection may help to explain the partial association of EBV in certain cancers and provide a mechanism for how this tumor virus can act in a "hit-and-run" fashion in some malignancies[10,17,22-25].

Table 2. Association between EBV and different types of cancer[a]

Latency type	Expressed EBV genes	Malignancy	Lines of evidence	% EBV positivity
0	EBER1,2 miRNAs		all EBV protein expression is silenced only the non-coding EBERs, BARTs and miRNAs are transcribed	
I	EBNA1	Burkitt lymphoma (Ig-myc)	Demonstration of monoclonal EBV genomes in tumor cells	>95% (endemic)
IIa	EBNA1, LMP1 (+/-), LMP2, BARF1[b]	Nasopharyngeal carcinoma	Elevated anti-EBV antibody titers before and at diagnosis	>95% cellularity)
		B- and NK/T-cell lymphoma	Demonstration of monoclonal EBV in tumor cells	(endemic) 70% (mix
		Hodgkin lymphoma	Increased risk in IM patients*	>90%
IIb	EBNA1 LMP2, BARF1[b]	Gastric carcinoma DLBCL[c] (elderly) CLL[d] ("hit-and-run")	Demonstration of monoclonal EBV in (a subpopulation of) tumor cells Increased risk in patients with (epi)genetic and EBV-serological abnormalities	>90% (LELC)[e]
III	EBNA1-6, LMP1, LMP2	Post-transplant lymphoproliferative disorders	Direct outgrowth of EBV carrying B-cells into malignant lymphoma	>90%
		AIDS-related lymphomas	Overall risk correlates with immune disfunction	>95%

[a]Summarized from references 1,9,10,24. [b]only carcinomas. [c]Diffuse large B-cell lymphoma. [d]Chronic Lymphocytic Leukemia [e] Lymphoepithelioma-like carcinoma. *Prior IM is associated with an increased risk of developing HL.

EBV moves between host lymphoid and epithelial cells, switching its tropism while enhancing persistence and evading immune responses[10]. Essentially all undifferentiated NPC and EBVaGC are representative epithelial malignancies associated with EBV[3,4,11]. In epithelial cells, the limited subset of virus latent proteins (EBNA1 and LMPs; latency type II) contribute to the malignant phenotype, either as tumor initiator, or more probably, as tumor progressor[11,12]. Trafficking of virus from B cell to epithelial cell and back is then not only important for persistence – when this balance is disturbed as shown by elevated antibody titers against virus lytic replication proteins – but it may also accelerate the development of NPC[10,26]. Genetic susceptibility and environmental condition (food, inflammation) that trigger EBV into replication may contribute to enhanced epithelial infection and carcinogenesis[26].

The hypothesis that EBV enters an already premalignant cell and moves it toward malignancy may also be true for EBVaGC case[2,5]. Chronic gastritis might enhance the interaction between gastric epithelial cells and B lymphocytes, and cytokines produced by inflammatory cells might support the growth of EBV-infected gastric epithelial cells[27-29]. Differences in individual inflammatory responses by either genetic and/or environmental effects, possibly affect the oncogenic pathway leading to EBVaGC[28]. Strong DNA methylation-mediated repression of tumor suppressor genes driven by virus further promote monoclonal growth of EBV infected cells[27,28,30].

EBV can be considered as the prototype of oncogenic virus that behaves as a direct transforming agent[11,13]. Most clearly, this is seen in the transplant setting where immune suppression allows latently infected EBV-positive B cells to grow into malignant lymphoma[31]. In classical EBV-associated cancers (undifferentiated NPC, BL, HL), the virus genome is present virtually in all neoplastic cells, showing the expression of its RNAs and proteins that varyingly contributes to the induction of transformed phenotype[1,3,4,6,31]. However, the presence of homogeneous (clonal) EBV episomes detected in several EBV-associated cancers as well as in some pre-neoplastic lesions suggested that these tumors develop from a single cell that was infected by EBV before the outgrowth and are consistent with a role for EBV in the early phases of tumor development[1,3,31,32].

Besides behaving as a direct drive of neoplastic progression, EBV could also cause the development of other type of tumors such as NK/T cell lymphoma, diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL) and smooth muscle tumors (SMT) [3,13,33-35]. These tumors might have previously not been linked to EBV because they do not carry the viral genetic material. An abnormal immunological response against EBV in patients with CLL and DLBCL has been evidenced, however EBV markers such as EBER1 and LMP1 are detectable in only a subpopulation of these tumor cells[35-37].

The presence of EBV within tumor microenvironment could also indirectly contributes to the malignant evolution by enhancing the production of cytokines, suppressing the local immune through the activation of viral oncogene, LMP1 and driving tumor proliferation via secreted exosomes containing viral products such as LMP1 and EBV-regulated miRNAs[11,12,38,39]. This way EBV potently inhibits both cytotoxic T lymphocyte and natural killer cell responses[2,10,31]. Overall, the number and diversity of EBV-associated malignancies worldwide are still increasing. It involves multiple cell types and oncogenic events, directly or indirectly all leads to enhanced tumor cell growth, apoptosis resistance, metastatic behaviour, immune escape and modulation of the microenvironment, with a "hit-and-run" mechanism as an ultimate feature[6,9,19,24,26,31,32,38].

The role of EBV in NPC

Non-keratinizing undifferentiated NPC (classified as WHO type III) is nearly 100% associated with EBV, whereas keratinizing NPC only demonstrates such strong association in high-incidence areas including Southern China, Southeast Asia, the Middle East/North Africa[40]. Undifferentiated NPC is rare in most other parts of the world, however incidence of NPC is increasing in low-risk populations such as Europe and USA due to worldwide migration[41]. The stable maintenance of the EBV genome in epithelial cells requires chromosomal (epigenetic) alterations leading to upregulation of cyclin D1 and hTERT[17,25,42].

In NPC, DNA methylation and modification of histones within the EBV genome itself play important roles in type II latency program by restricting the expression of EBV EBNA1, LMP1, LMP2 and BARF1 proteins[38,42,43]. Dedifferentiation of epithelial nasopharynx by EMT (epithelial-mesenchymal transition) reprogramming via LMP2A, epigenetic silencing of tumor suppressor genes or mutation are hypothesized to play a role in tumorigenesis and provoke progressive metastasis[43,44]. Investigations to identify and characterize NPC cancer stem cells (CSCs) are still ongoing. LMP1 and BARF1 are considered the major EBV oncogenes that have transforming potentials in the epithelial background, however the exact oncogenic mechanism of the virus in NPC tumorigenesis remains enigmatic[2,8,17,43].

Although EBV infection is associated with immune infiltrates, viral factors repress the activity of antitumor lymphocytes, contributing to NPC tumor survival[26,40]. Environmental risk factors such as alcohol consumption and tobacco smoking observed in populations outside endemic regions is reported to be associated with NPC[26,41,43]. Notably, emerging integrated approaches by means of whole-exome and targeted deep sequencing, as well as single nucleotide polymorphism (SNP) array analysis identified several key genomic alterations including epigenetic defects and suggested biological relevance of these genetic changes to the NPC malignant phenotype[43]. Molecular alterations, including

chromosome loss, are seen in epithelial tissues in ethnic groups at high risk for NPC[1,4,26,43]. Familial clusters of NPC between different ethnic subpopulations in endemic regions and migrant studies indicate that genetic susceptibility and lifestyle factors determine the risk of developing NPC[4,5,41]. Report showed that anti-EBV seropositivity production shared by family members correlates with increased risk for NPC[1,4,5]. Taken together, EBV infection, environmental factors and genetic mutations targeting cellular modulators of epigenetics appear to play integral roles in the modulation of NPC development and progression[26,40].

Whilst primary NPC especially at early stage can be treated with radiation alone or with chemotherapy resulting five year survival rates of 50 to 70%, nasopharynx and cervical lymph node recurrence still occurs in 12 to 22% of patients who underwent standard chemoradiation treatment during 5 years[40,43,45]. Both local and regional recurrent NPC are frequent in differentiated (18.7%) rather than undifferentiated type (8.7%)[44]. Relapse and early metastasis in patients with NPC are still the common causes of mortality[40,45]. The mechanisms involved in therapeutic resistance and tumor recurrence have not yet been fully understood and mostly remain unexplained.

EBV approaches for molecular detection of NPC

Almost all patients with NPCs had detectable IgA responses to the EBV capsid antigen (IgA-VCA) and EBV nuclear antigen 1 (IgA-EBNA1) whereas such elevated antibody levels are rarely seen in healthy controls or in patients with other head and neck tumors[4,40,46]. Many patients with NPCs also have responses to early antigen (IgA-EA) and EBV-specific DNase antibodies. ELISA assays based on purified recombinant EBV antigens or defined synthetic peptides are therefore useful as screening or diagnostic test[47-49].

Circulating EBV-DNA analysis may also convenient for early detection of NPC[40,43,50]. Plasma viral load might be superior to whole blood DNA in a surveillance program[50,51]. It may indirectly reflect disease burden because they are released by cancer cells [26,40,52]. However, whether plasma DNA copies are superior to IgA EBV titer for population screening is controversial at best[50,51]. Furthermore, plasma EBV-DNA and/or VCA-IgA are believed to be indicators for NPC survival[50,52]. EBV biomarkers have been identified in the circulation of patients with NPC, including proteins (LMP1 and BARF1) and non-coding RNA molecules (EBER1, EBER2, and BART miRNAs)[43,52]. Molecular tests relying on in situ EBV-DNA and -RNA detection (within tumor cells) and reflecting tumor activity are fundamentally important for NPC diagnosis at the primary site and loco-regional recurrence.

An adequate tumor biopsy is required to perform EBV-encoded RNA in situ hybridization (EBER-RISH), which is the golden standard in clinical laboratories for defining a lesion as EBV-related. EBERs are reliably expressed in all latently infected cells in every benign and

malignant lesion[40,43,52]. Endoscopy examination and viral DNA load in nasopharyngeal brushing could be an option to represent tumor status in addition to pathological examination[53-56]. It is established that monoclonal EBV-DNA is present in infected carcinomas. It was suggested that nasopharyngeal brush or swab could be the alternative method for effective viral gene detection within tumor cells[53-55]. Nasopharyngeal brush / swab sampling combined with EBV-DNA measurement in nasopharyngeal brushing demonstrated suitable use for detecting patients with NPCs at early stage due to its original lesion at the site of the nasopharynx. The potential of DNA load quantification in nasopharyngal brushing has also been explored for detecting locally recurrent NPC[52,56].

Despite its capacity to detect disease recurrence and metastases, the molecular nature of circulating EBV-DNA has been identified as free DNA fragments, and it was not contained inside intact virions[40,43,52]. Although recent discovery demonstrated that healthy, living cells could release DNA and RNA such as intact virions produced by viral replication that may also enter the circulation, several studies suggesting that the viral DNA in the circulation of patients with NPCs is indeed released by NPC tumor cells upon apoptosis of dead or dying cells rather than through viral replication.

In recent years, there has been increasing interest in the presence of different viral and cellular miRNAs in EBV-infected B cells and epithelial cells. EBV is able to produce its own miRNA consisting of BART and BHRF clusters that encode 44 mature miRNAs[6,8,56,57]. Several EBV-encoded BART miRNAs are highly expressed in NPC lesions suggesting the pathogenesis of NPC is not only regulated by EBV-encoded proteins but EBV-associated miRNAs also play an important role in NPC development[57,58]. Interestingly, unlike the BART-encoded miRNAs, BHRF1-miRNAs do not seem expressed in EBV-associated NPC tissues[43,58,59].

A recent study identified variable high plasma levels of two EBV-BART miRNAs (EBV-miR-BART7 and EBV-miR-BART13) in patients with NPCs, whereas absent or low expression was marked in the plasma of both non-NPC and healthy patient controls. Importantly, higher levels of EBV-miR-BART7 were not only associated with a more advanced disease stage, but also provided a 90% predictive confidence of NPC outcome in patients who underwent radiotherapy[6,60].

In another series, elevated levels of EBV-miR-BART1 were associated with advanced clinical stages of NPC and demonstrated an effective marker for NPC diagnosis[5,61,62]. Other studies demonstrated the potential of EBV-miR-BART17 as a post-treatment biomarker for prediction of recurrent NPC and for monitoring tumor dynamics[63]. However, translation of viral miRNA markers to clinical setting remains a considerable

challenge, thus further exploration using different populations is required to determine the viral miRNA potentials and their relevance in NPC.

The EBV-encoded miRNAs may enhance latency by targeting host transcripts[6-8,58]. For instance, EBV-miR-BART9 was observed to target E-cadherin. It enhanced migratory ability and invasiveness of NPC cells that contributes to the aggressiveness of tumor cells[57]. In both EBV-positive NPC tissues and cultured cells, miR-BART9 is expressed at levels higher than miR-21, an endogenous miRNA with known oncogenic activities (oncomiR). Essentially, predictive signatures of EBV-miR-BART9 and EBV-miR-BART10 for NPC have been directly associated with enhanced tumor initiation or progression, suggesting poor survival of patients with NPCs[6,57]. These predictive miRNAs are also believed to be responsible for driving tumorigenesis.

Another study performing differential expression analysis in samples from patients with NPCs vs. patients with non-malignant nasopharyngitis found that EBV miRNAs (miR-BARTs) were more than twice the number of endogenous human miRNAs (62.5% vs. 29.2%, respectively)[6]. Roles for EBV-encoded miRNAs in the regulation of both viral and cellular transcripts have been fully described, and much more work is required to characterize the function of these non-coding RNAs.

Genomic diversity of EBV and its relevance to NPC diagnosis and treatment

EBV genome is divided into type 1 and type 2 mainly based on patterns of genetic divergence of the EBNA2 and EBNA3 genes[6,26,34,64]. EBV types have differ geographical distributions. Type 1 strains are being more prevalent worldwide and have greater transforming potential whereas type 2 strains are being locally widespread in Africa[4,6,34,64]. Strain B95-8 showing a 12 kb deletion in BARTs compromising 17 of the pre-miRNAs is a representative type 1 EBV prototype and widely used as reference[4,34,64]. Although there is a high level of similarity between EBV strains, variations exist in some viral genes, mainly in the latent genes, that give rise to functional differences. Amino acid polymorphism within EBNA1, LMP1 and RPMSI genes and the BARTs family indicates that these sequence variations might contribute to NPC susceptibility[6,39,65-67]. Taken together, high-throughput sequencing technology revealed the existence of substantial genetic variation in EBV genome, nevertheless its contribution to malignancy remains unclear.

Phylogenetic analysis in primary NPC biopsy specimens implied that Asian strains of EBV, particularly from China, Indonesia and Japan, are unlike EBV strains from other parts of the world[4,6,66,67]. Furthermore, genomic diversity data discovered that the endemic strain of EBV in Southern China is inherently associated with NPC[66]. Thus, it is possible that a specific EBV variant is involved in that cancer[64-67]. Genome wide comparison of

EBV genomes isolated from patients with NPCs and normal subjects identified natural variation in EBV-DNA sequence, perhaps in relation to immune escape in context of certain MHC types. For example, the loss of heterozygosity (LOH) at chromosomes 3p and 9p that occurs as an early event[4,6,43,64,65]. Therefore, for NPC in Southeast Asia, natural variation in EBV, genetic variation of the host, and local environmental cofactors (including co-infections) are not only serve as geographical markers but also maybe factors to consider when designing NPC vaccine[5,6].

Lin and colleagues examined the chromosomal changes in 128 NPC cases and they suggest altered chromatin remodeling possibly be an oncogenic feature of EBV infection in neoplasia[68]. Moreover this information will ultimately point to new therapeutic strategies. Although EBV-associated NPC results in a relatively low level of genomic alteration and EBV itself is a tantalizing target, mutations in the chromatin-modification pathway linked to EBV burden may serve as a potential alternative. Carcinomas with these mutations might be sensitive to drugs such as histone deacetylase inhibitors (HDACIs)[11,34,68].

In recent years, research on different viral and cellular miRNAs of EBV-infected B cells and epithelial cells has been increased[6,8,26,58]. Roles for EBV-encoded miRNAs in the transcriptional regulation of both the viral and cellular genome have been described, but much more work is required to characterize the function of these RNAs[8,43]. Hooykaas and co-workers recently demonstrated a depth assessment of the expression levels and silencing activity of all EBV miRNAs in B- and epithelial cell lines of different latency stages[69]. Their arguments are not against the important role of cell-specific or virus-specific factors in regulating miRNA activity or its abundance, but rather suggest the importance of (pri-)miRNA processing in determining functional expression levels[66,69]. In conclusion, the role of EBV strain variation in NPC tumorigenesis which was previously poorly understood is becoming more tractable with the revolution in virus genome technologies.

All aspects of EBV research and clinical intervention rely on basic definition of the virus genome, understanding the global picture of what is wild-type EBV, how it may vary and how that might be relevant to disease[70]. For example to make an effective EBV vaccine it is important to ensure that its efficacy will include natural variants of the virus that may be present in different populations or parts of the world. Research on EBV strain variation will also be relevant to the successful cytotoxic T cell (CTL) therapy of post-transplant lymphoma caused by EBV[3,4,34]. CTLs recognise epitopes in EBV EBNA and LMP genes and variation in them is likely to affect the success of CTL therapy[34]. It will also be important to understand strain variation to ensure detection of the correct EBV

sequence in qPCR viral load assay for screening and diagnosis of NPC[70]. Taken together, technological advances in sequence analysis provide new opportunities for future NPC research and treatment.

Targeted therapy for EBV-associated carcinomas

NPC is radiosensitive and therefore radiotherapy is the primary and curative treatment choice for stage I NPC. Unfortunately, this stage is rarely observed upon clinical presentation and patients usually have more advanced stages of disease when they first come to clinic. Patients with locally advanced disease (stage II through IVB) receive concurrent chemoradiotherapy with cisplatin[3,40,71,72]. Although current treatments for NPC have improved the 5-year survival rate to 50%, metastatic disease for which there is no curative therapy eventually develops in 20% to 25% of patients[3]. Persistence of residual disease after primary treatment leads to high NPC mortality; in these cases the best management remains to be determined[40,44,71-76]. Table 3 summarizes the novel targeted therapies for EBV-associated carcinomas.

Since EBV is present exclusively in every NPC tumor and rarely in normal cells, various approaches have been developed to target EBV in NPC cells[71,72]. Advanced understanding of EBV cancer pathobiology provides the rationale for emerging treatment strategies that include activation of lytic viral infection combined with antiviral drugs, inhibition of EBV-induced oncogenic cellular signaling pathways, adoptive EBV-specific T-cell therapies, and EBV vaccines (Table 3)[5,34,71-76]. Intensity-modulated radiotherapy *(*IMRT) with or without chemotherapy produces excellent locoregional control and it is preferred for NPC.[37,67]

Although numbers of clinical studies have explored the effect of EBV-targeted therapy alone or in combination with chemotherapy, its application in NPC remains in its infancy compared with its successful use in PTLD and lymphoma[1,3,34,72,73]. Likewise, specific therapy is currently unavailable for patients with EBVaGC despite its distinct biological characteristics from EBV-negative GC. In centers where modern radiation technology (IMRT) is limited or unavailable, disease control and survival in patients with NPC and EBVaGC is low and becoming unmanageable[77]. Therefore, given its distinct biologic characteristics, viral enzyme-targeted antitumor strategy, i.e. so called cytolytic viral activation therapy (CLVA), may potentially provide an affordable alternative for treating patients with NPCs in these regions[72].

CLVA that includes EBV lytic replication coupled with cytotoxic anti-EBV drugs has been tested recently in phaseI/II clinical trials of NPC and was also introduced in preclinical studies of EBVaGC[78-81]. This approach has provided some clinical benefit in certain highly

Table 3. Novel targeted therapies for EBV-associated carcinomas[a]

Modalities	Classification	Preclinical studies / clinical applications	Comments
Drug treatment	Cytotoxic drugs	NPC EBVaGC	Acyclovir/Ganciclovir (effective only against lytic EBV infection)
	Antibody-therapy	NPC	anti-EGFR[b] (nimotuzumab) anti-VEGF[c] (Bivacluzumab/Avastin)
	Inhibitors of EBV-activated signaling pathways	NPC NPC NPC EBVaGC	Akt inhibitor MK-2206 (LMP1, LMP2 activation of PI3K/Akt/mTOR) Bortezomib (LMP1 activation of NF-κB) Brentuximab vedotin (CD30 signaling)
Immunotherapy	Vaccination	NPC	Recombinant modified vaccinia virus ankara EBNA1/LMP2 Adenoviral vector carrying a truncated form of LMP1 and LMP2 CTL epitope peptide-based vaccine
	Treatment of disease	NPC	EBV-specific CTL-based[d] / TIL-based[e] adoptive therapy Chimeric antigen receptors (CAR) therapy
Virus-directed approaches	Targeting EBV episome	NPC	Low dose hydroxyurea treatment
	Inhibition of EBV transforming protein	NPC	Antisense RNA against LMP1 oncoprotein; small molecule inhibitors of EBNA1-DNA binding
	EBV-dependent expression of cellular toxins	NPC	Expression of detrimental cellular proteins through OriP dependent expression vector
	Induction of EBV lytic replication	NPC / EBVaGC EBVaGC NPC NPC	Phenylbutyrate, arginine butyrate, SAHA and other HDAC inhibitors 5-Aza, cisplatin, 5-FU, taxol Gemcitabine + valproic acid Bortezomib (± gemcitabine)
	Targeting EBV latent genes	NPC	CRISPR/CAS9 mediated genome editing

[a] summarized from references 2,3, 71-76. [b] epidermal growth factor receptor. [c] vascular endothelial growth [d] factor cytotoxic T-lymphocytes. [e] tumor-associated lymphocytes

selected patients[78,79]. The rationale for its use is the effective phosphorylation of cytotoxic drugs (acyclovir or ganciclovir) to kill tumor cells containing lytic EBV infection. During drug-induced EBV reactivation from latency (Table 3), the virus expresses two critical kinases i.e. EBV thymidine kinase (TK) and the BGLF4 gene product, protein kinase (PK) that can effectively activate/phosphorylate the antiviral drug, allowing their functions as cytotoxic DNA chain terminators.

In addition, lytic reactivation induces de novo higher EBV antigen expression on tumor cell membrane leading to improved immune elimination[34,72,79]. Interestingly, oral formulation of lytic induction agents (gemcitabine, valproic acid and valganciclovir)

went off-patent, thus the price of these drugs is affordable for patients in the regions with limited healthcare facilities. Furthermore, this alternative therapeutic strategy could limit the need for advanced radiotherapy facilities. In conclusion, CLVA merits further research and clinical evaluation as alternative strategy to selectively eliminate EBV-carrying tumor cells in the treatment of EBV-associated carcinomas.

Outline of the thesis

Survival rates differ significantly between NPC patients in early and late stages. Because symptoms related to NPC in the early stage are usually nonspecific, improve early detection of NPC and appropriate management are important to achieve favourable treatment results. This thesis is focused on defining EBV-associated biomarkers for diagnosis and prognosis of NPC and using virus itself as a potential target in cancer therapy.

Chapter 2 reports the collection of NPC cases in Indonesia and measurement of EBV-DNA in whole blood and nasopharyngeal brushings of patients with NPCs before and after treatment. EBV IgA serology markers are analysed as well. This study indicates the potential of nasopharyngeal brush sampling combined with EBV-DNA quantification for early NPC screening and disease monitoring. **Chapter 3** identifies the expression levels of EBV miRNAs in the serum and nasopharyngeal brushings of patients with NPCs and healthy subjects. This study concludes that the detection of stable circulating BART miRNAs in serum of patients with NPCs allows non-invasive NPC diagnosis. Results in nasopharyngeal brushings raise the potential of EBV miRNAs as novel diagnostic marker. **Chapter 4** defines EBV mRNA profiles and viral DNA methylation status in nasopharyngeal brushings. This study indicates that nasopharyngeal brush is not only useful as a diagnostic tool for NPC but also reliably represents tumor origin. **Chapter 5** describes the use of multi-target plasmid pool for quantification of cDNA, mRNA detection and standardisation. Multi-target plasmid pool system uses gene specific primers that is applicable for gene expression analysis and useful for defining latency types in EBV cell lines and specimens from patients suspected of EBV-associated diseases. This study contributes to a better understanding of EBV infection in patients with NPCs undergoing virus reactivation therapy. **Chapter 6** explores the therapeutic potential of curcumin-derivatives (curcuminoids) as putative EBV lytic activators for improved treatment of EBV-associated carcinomas. Curcuminoids promoted EBV lytic induction by gemcitabine and valproic acid (GCb+VPA) in EBV-positive NPC and EBVaGC cells when added simultaneously and thus may serve as adjuvant for CLVA treatment. Overall discussion and future prospects are described in **Chapter 7**.

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2

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 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: Nasopharyngeal 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 nasopharyngeal brushing was well tolerated by patients and revealed high viral load in the 228 NPC cases at diagnosis than 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: Nasopharyngeal 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 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 brushing plus EBV-DNA load as tumor marker gives excellent diagnostic and prognostic results compared with the biopsy. The nasopharyngeal brush approach proved better than EBV-DNA load assessment in blood and EBV-IgA serology. The data suggests that nasopharyngeal 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 nasopharyngeal 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 nasopharyngeal brush appears well suited for use in NPC screening in high incidence regions, like Indonesia.

Introduction

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 per 100,000[2]. In the Dr. Cipto Mangunkusumo Hospital, (Jakarta, Indonesia), NPC is the fifth 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 III and IV), 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 nonendemic 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 NPCs 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 noninvasive 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 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 patients with NPCs before and after treatment may be valuable for assessment of disease progression[17], as levels of EBV-DNA in the circulation of patients with NPCs 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 posttreatment management.

The present study evaluates the diagnostic and posttreatment value of viral DNA load measurement in minimal invasive nasopharyngeal brushings and in parallel in whole blood samples collected at diagnosis and 2 months after start of therapy in 228 patients with advanced NPCs. The viral load was compared with 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 (Jakarta, Indonesia) with suspected NPCs during 2006-2009 were enrolled into this study. About 20% of the patients were referred by regional health centers where initial diagnosis was conducted. 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 lesion by fiber optic nasopharyngoscopy with photography and computed tomographic (CT) scans. In all patients, a nasopharyngoscopy-guided nasopharyngeal brushing was performed first, followed by biopsy from the same area of the suspected NPCs. Endoscopic findings were classified as normal (no tumor), suspicious tumor, or clearly abnormal.

Of the 289 patients at intake, 228 had biopsy proven NPCs 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 areas 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) in 116 of 228 patients from whom an adequate biopsy specimen was available.

Table 1. Description of patients population used for validation of viral load by nasopharyngeal 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. The number of samples of patients with NPC taken at diagnosis and after 2 month follow-up

Patients with NPC	Nasopharyngeal brushing	Whole blood	Serology
At diagnosis	208	149	174
After 2 months follow-up	69	65	68

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-fluorouracil (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. Because of undercapacity of radiotherapy and the poor financial situation of most patients, optimal treatment, that is full chemoradiation, was not always feasible and different treatment protocols had to be implemented.

Sampling procedures

Nasopharyngeal brushing was performed under rigid or flexible endoscopic guidance by experienced ENT specialists and ENT resident trainees. Endoscope-guided nasopharyngeal brushings were performed under local anesthesia (1% Lidocaine spray, Astra Zeneca). 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) was used in combination with a plastic catheter covering the entire brush to prevent contamination by cells from non-nasopharyngeal sites. The catheter covering the cytobrush was inserted via the nose until the nasopharyngeal cavity was reached. Subsequently, the brush was released from the catheter and the cytobrush was rotated several times over the nasopharyngeal 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) mixed well and stored in 1-mL aliquots at –80^{0}C until use[14,20].

In all NPC suspected patients, nasopharyngeal 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 nasopharyngeal 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 to 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 Center, 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 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 (EBNA1), 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 S1).

Primers, probes and PCR conditions have been described in detail previously[14,22]. Cutoff value (COV) for EBV-DNA load in nasopharyngeal brushings was defined at 2,300 copies per 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 2,000 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 (PPV and NPV).

The amount of human diploid genome equivalent in NP brushing specimens was determined by quantitative LightCycler-based polymerase chain reaction (LC-PCR) targeting a 197-bp fragment of the human β-globin gene[23].

EBV serology

Serum samples from patients with NPC, 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].

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

Table 3. Continued

		Number	Percentage (%)
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	Complete response	52	22,8
at 2 months posttreatment	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

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 NPCs at intake. $P<0.05$ 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 2 groups (procedures) and for analysing the level of comfort of the conducting a nasopharyngeal 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 nasopharyngeal brush and whole blood at diagnosis and after treatment of the paired samples was conducted 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, nonmalignant 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 III. Although the age of the majority of patients with NPCs (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 nasopharyngel brushes, 149 whole blood and 174 serology samples could be evaluated at diagnosis. Posttreatment nasopharyngel brush samples of 69 patients were analyzed as well as 65 parallel whole blood and 68 serology samples.

Viral DNA load in nasopharyngeal 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

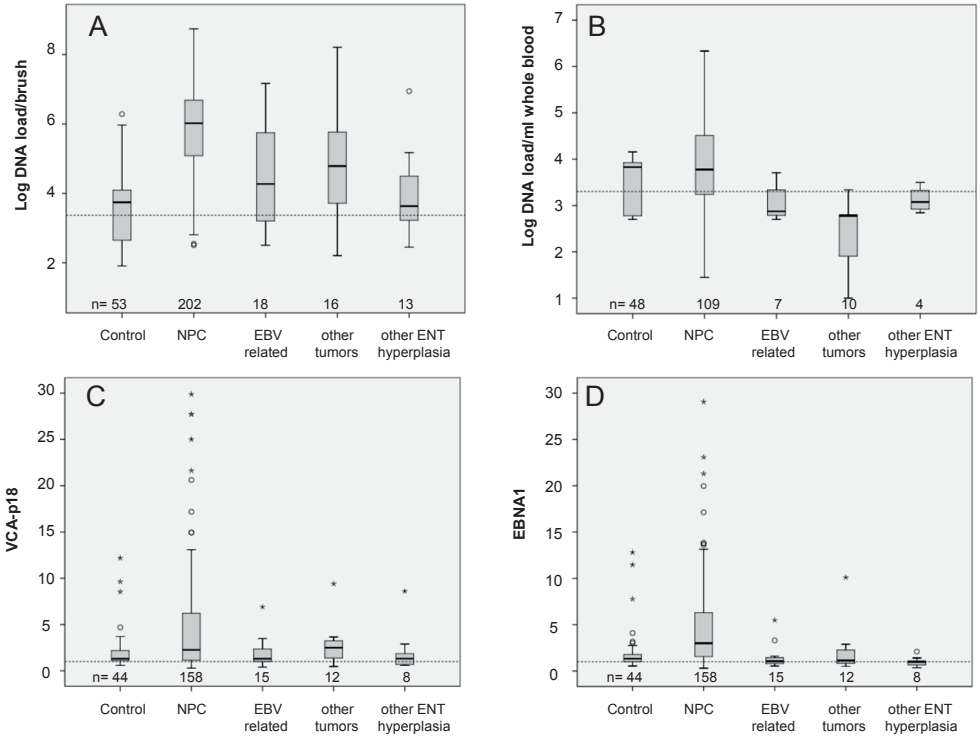

Figure 1. EBV parameters at diagnosis. A, Viral DNA load in nasopharyngeal brushings (log scale). The EBV-DNA levels observed were significantly different between NPC and healthy controls, EBV-related malignancies ($P < 0.001$), and other ENT disorders ($P < 0.001$), whereas a near significant difference was found between NPCs and non-NPC head & neck cancers ($P = 0.059$). B, viral DNA load in whole blood (log scale). The mean EBV-DNA load in blood was not significantly different between NPCs and healthy controls ($P = 0.601$), EBV-related malignancies ($P = 0.109$), and other ENT disorders ($P = 0.401$), whereas NPCs and non-NPC head and neck cancers did show a significance difference ($P < 0.001$). C, IgA VCA-p18 serology. EBV-specific VCA-p18 IgA serology was significantly higher in NPC vs. healthy controls ($P = 0.011$), but not between NPCs and EBV-related malignancies ($P = 0.21$), non-NPC head and neck cancers ($P = 0.75$), and other ENT disorders ($P = 0.57$). D, EBNA1-IgA serology. EBNA1-IgA serology was significantly higher in NPCs vs. healthy controls ($P < 0.001$), and EBV-related malignancies ($P = 0.018$), and was close to significance for NPCs vs. non-NPC head and neck cancers ($P = 0.20$), and other ENT disorders ($P = 0.054$).The dotted line in each graph represents the cutoff value for each assay, as defined in the methods section.

analyzing the EBV-DNA load in nasopharyngel brushings taken at diagnosis. Clinical cut-off value (COV) for viral DNA load in nasopharyngeal brushings was previously defined at 2,300 EBV-DNA copies per nasopharyngel 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.

Table 4. Sensitivity, specificity, PPV and 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 nasopharyngel brushings from patients with NPCs showed significant higher levels of viral load than controls (median NPC, $1.0x10^6$; range 0 x 10^8 to 1.9 x 10^8 vs. median, 4.0 x 10^3; range 0 x 10^5 to 1.2 x 10^5; $P < 0.0001$).

EBV-DNA was detected above COV in 95% of NPC cases, confirming NPC tumor cell presence. The higher EBV-DNA values in nasopharyngel brushings in the "other" tumor group compared with 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 nasopharyngeal 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 ($P = 0.60$, 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 patients with NPCs at diagnosis was significantly higher than the clinical COV of 2,000 copies/mL whole blood[21] compared with the control groups and even compared with 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

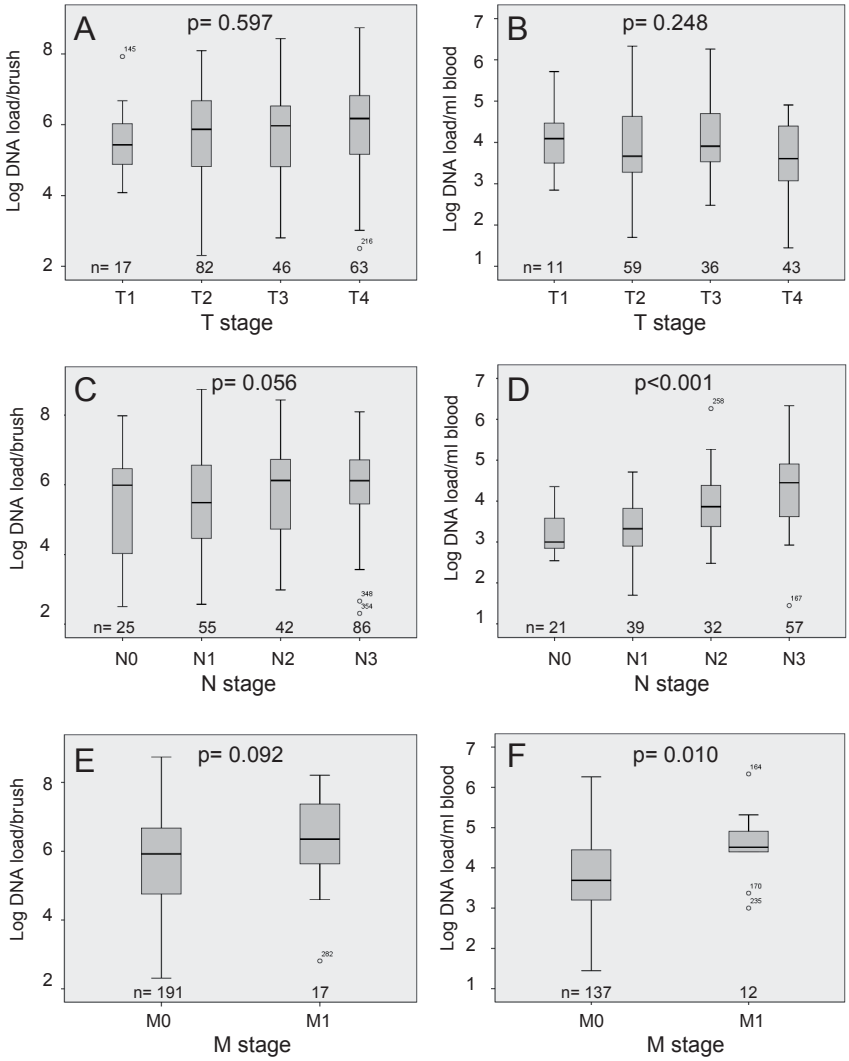

Figure 2. EBV markers in relation to tumor characteristics at intake. A, Correlation of EBV-DNA load in brush with T stage (DNA copies in log scale), showing no relation using ANOVA ($P = 0.597$). B, Correlation of EBV-DNA load in whole blood with T stage ($P = 0.248$). C, Correlation of EBV-DNA load in brush with N stage ($P = 0.056$). D, Correlation of EBV-DNA load in whole blood 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 ($P = 0.010$).

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 $P = 0.01$, respectively; Figure 2D and F).

Serology IgA-VCAp18 and IgA-EBNA1

IgA-VCAp18 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), than in healthy controls ($P = 0.001$). Sera form 79.8% of the patients with NPC had IgA-VCAp18 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 VCAp18 compared with patients with NPCs 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 than in other groups resulting in 85.6% of the patients having IgA EBNA1 responses above the COV. Patients with NPCs have higher median value (median, 3.0; range, 0.3–29) than 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 VCAp18-IgA or EBNA1-IgA antibody levels in ELISA and TNM staging of the NPC tumor at intake (data not shown).

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 VAS. The brush procedure was characterized by a median VAS score of 5 (range, 3–6), which is significantly less compared with 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 3 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 nasopharyngeal brushings from both sides of the nasopharyngeal cavity, that is, at and

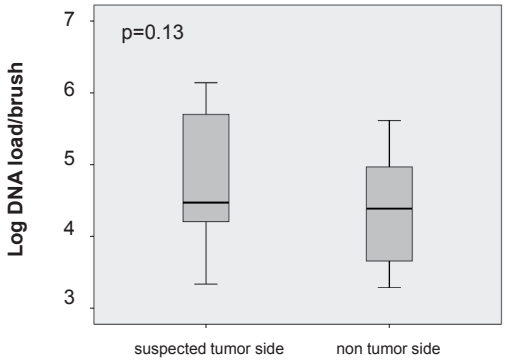

Figure 3. Viral DNA load of bilateral side nasopharyngeal brushing. Bilateral brushing (n=20) was performed at the site of suspected tumor location and the nontumor site. The mean viral DNA load was 3.4 x 10^5 vs. 7.1 x 10^4 copies per brush, respectively (P = 0.13), which indicates that single brushing at the site of enlarged neck node may be more representative for detecting NPC presence.

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 copies per brush; mean, 188.782 copies per brush; range, 4.14 x 10^2 to 4.7 x 10^6 copies per brush) than in the opposite site (48%>COV; median, 2.400 copies per brush; mean, 43.258 copies per brush; range, 0 x 10^6 to 1.1 x 10^6 copies per 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 nonlesional side of the nasopharynx were still significantly higher that the EBV-DNA load observed in non-NPC tumors and ENT hyperplasia (P < 0.001).

Comparison of viral DNA load at diagnosis and 2 months posttreatment

In 69 patients, the effect of the therapy on the viral load was analyzed by comparing the viral DNA load in nasopharyngeal brush and whole blood at diagnosis and 2 months posttreatment. The median EBV-DNA load in nasopharyngeal brushing at diagnosis was 9 x 10^5 copies per brush and decreased after 2 months posttreatment to a median of 3 x 10^3 copies per brush indicating a 300-fold reduction (Figure 4).

Initially, 96% of patients had a viral load > COV level in the nasopharyngeal brush, but after treatment this dramatically reduced to 39.4%. Similarly, the level of EBV-DNA in whole blood was significantly lower posttreatment 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 nasopharyngeal 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.

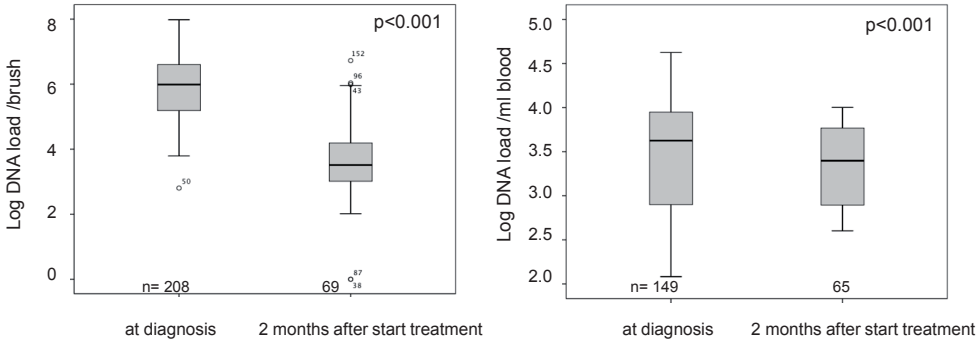

Figure 4. Viral DNA load in nasopharyngeal brush and whole blood at diagnosis and after treatment. A, EBV-DNA load in nasopharyengal brushings (copies per brush). B, EBV-DNA load in whole blood. There was a significant decrease in EBV-DNA load in both nasopharyengal brushings and whole blood at diagnosis compared with 2 months posttreatment for samples paired before and after treatment ($P < 0.001$).

Patients treated with neoadjuvant plus hyperfractionated radiotherapy had a median value of 8.9 x 10^6 copies per brush at diagnosis that decreased after therapy to 1.7 x 10^5 copies per brush ($P = 0.006$). Patients treated with concurrent chemoradiation had a median DNA viral load in brushings of 1.9 x 10^7 copies per brush at diagnosis decreasing to 5.8 x 10^4copies per brush after therapy ($P = 0.049$). Only one patient with neoadjuvant and radiotherapy had increased DNA viral load brush posttreatment (9.5 x 10^5 copies per brush) and one patient had increased viral load in whole blood at 2 months posttreatment, both linked to progressive disease.

Based on response to treatment, 41 patients had a complete response at 2 months posttreatment as judged by clinical examination plus a negative CT scan and negative biopsy. These patients had a posttreatment median viral DNA load in the nasopharyngeal brushing of 3.0 x 10^3 copies per brush, a significant difference compared with the pretreatment value of 1.7 x 10^6 copies per brush in this group ($P = 0.013$; Figure 5). In 22 patients with partial response, the median EBV-DNA load in nasopharyngeal brush pretreatment was 1.3 x 10^6 and posttreatment 3.2 x 10^3 copies per 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 starting treatment for both complete and partial responses.

Two of 3 patients with progressive disease posttreatment showed a median of EBV-DNA load in brush being below COV, whereas the posttreatment median level in blood was above COV in all 3. Two patients died within 2 months posttreatment 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

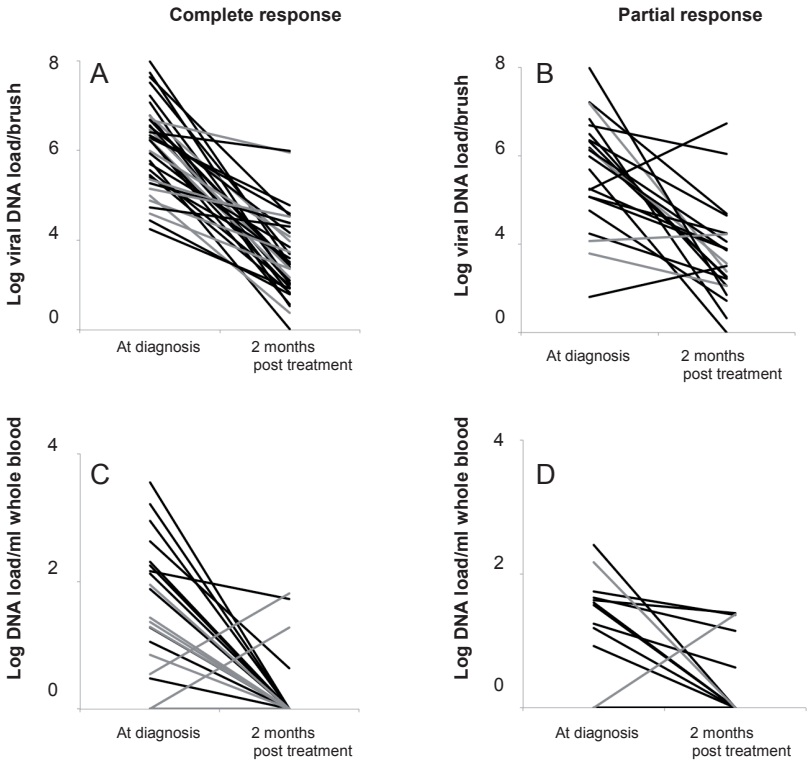

Figure 5. Viral DNA load of NPC patient samples before and 2 months posttreatment in relation with treatment response. Patients with NPCs treated with concurrent therapy are given in black and patients receiving neoadjuvant therapy are presented in gray. Presented is EBV-DNA load in A, in brushes of patients with NPCs with complete response; B, brushes of patients with NPCs with partial response; C, whole blood of patients with NPCs with complete response; D, whole blood of patients with NPCs with partial response. Complete response is defined as complete disappearance of locoregional disease by physical examination or X-Ray, CT scan, endoscopic examination and a negative biopsy at 2 months posttreatment; 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.

with 2 months after treatment, neither for VCAp18 nor for EBNA1 antibody levels individually, nor for different treatment regimens (data not shown).

Discussion

Pathologic examination for diagnosis of NPC requires an invasive biopsy that is painful and cannot be repeated easily. A less invasive diagnostic procedure by

using nasopharyngeal brush sampling would be preferred, also for assessment of posttreatment tumor activity. This nasopharyngeal 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 NPCs[25]. In this study, we evaluated minimal-invasive nasopharyngeal 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 showed that measuring EBV-DNA load in nasopharyngeal brushings provides a highly specific tool for primary NPC diagnosis with minimal patient discomfort, giving better sensitivity/specificity compared with EBV-IgA serology and EBV-DNA load in blood, as detailed in Table 4. Because most patients in this study presented with advanced-stage NPCs, the use of nasopharyngeal brushing for detecting early-stage NPC remains to be defined. In ongoing studies in patients with persistent head and neck complaints, nonresponsive to antibiotic or anti-allergy therapy, we are currently validating this method for identification of early-stage NPCs. The diagnostic utility of nasopharyngeal 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 NPCs.

Although detection of NPCs at early stage is important for the patient outcome, diagnosis is often difficult because of the nonspecific 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 patients with NPCs and patients with chronic head and neck complaints suggestive for early-stage NPC[25,26,27].

Nasopharyngeal brushings from patients with NPCs frequently contain extremely high levels of EBV-DNA compared with other clinical conditions, including EBV-related non-NPC head and neck cancers (Figure 1), confirming previous studies[28,29]. More than 95% of our patients with NPCs had a brush containing viral loads above COV. A negative result (5%) of brush EBV-DNA 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 nasopharyngeal brushing combined with quantitative real-time PCR directly reflects carcinoma-specific EBV involvement at the anatomical site of tumor development. The nasopharyngeal brush may greatly reduce the number of invasive nasopharyngeal biopsies required when applied for diagnosis and follow-up monitoring.

As bilateral brushing might be necessary for EBV tumor detection, Tune and colleagues originally recommend bilateral brushing as a routine to avoid missing small, localized tumors[13]. We conducted 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 nasopharyngeal 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 nasopharyngeal 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, nasopharyngeal 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 nasopharyngeal 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 predetermined clinical cutoff level in whole blood, being 71%, 50%, 86% and 20%, respectively (Table 2), were low compared with the nasopharyngeal 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 and colleagues in Hong Kong[17,18], as detailed elsewhere[22]. Pretreatment level of circulating EBV-DNA is considered to be a prognostic factor for NPCs[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 patients with NPCs 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 patients with NPCs. Some patients with extensive clinical disease (stage IVB) completely lacked circulating EBV-DNA, despite having high EBV-DNA levels in the nasopharyngeal 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 and colleagues found that T1 tumors had a significantly lower EBV-DNA level than 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 nasopharyngeal brushing at diagnosis and the clinical response at 2 months posttreatment. Therefore the initial EBV-DNA load values may not be taken as a prognostic marker.

At 2 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 nasopharyngeal 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. Posttreatment 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 nasopharyngeal brushings is a promising approach for NPC diagnosis and posttreatment monitoring and may reduce the number of invasive nasopharyngeal biopsies required. Although pathological examination for definite NPC diagnosis remains needed, molecular testing of nasopharyngeal brush material provides a promising and minimally invasive alternative requiring further validation. Nasopharyngeal 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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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary figure

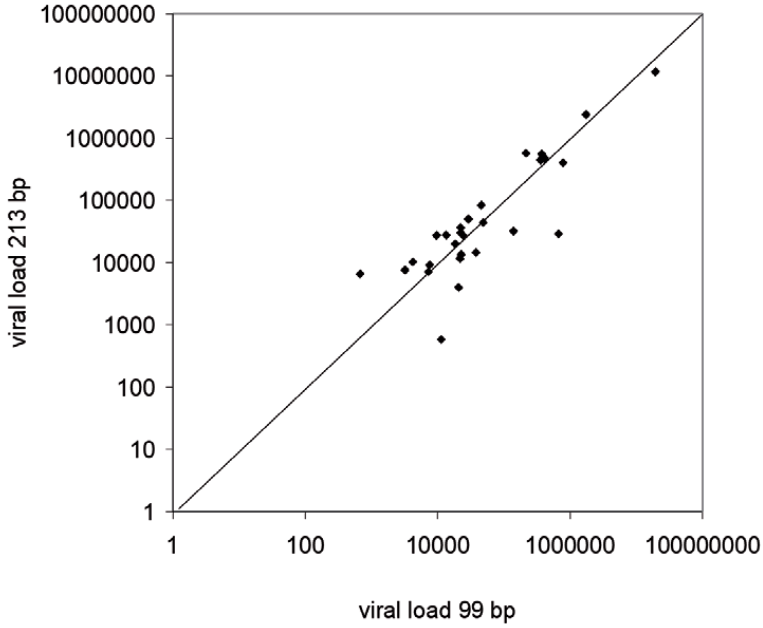

Figure S1. Comparison of the 99bp and 213bp PCR for measuring the EBV-DNA load in nasopharyngeal brushings. Both primer pairs gave similar results

3

Circulating Epstein-Barr virus miR-BART13 as a promising tumor marker for non-invasive early detection of nasopharyngeal carcinoma

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Submitted

Abstract

Introduction: Efforts toward early diagnosis targeting Epstein-Barr virus (EBV) gene products are of great importance to improve the 5-year overall survival rate in patients with nasopharyngeal carcinoma. Viral microRNAs are functionally involved in the pathogenesis of nasopharyngeal carcinoma and are detected in serum. Whether cell-free EBV microRNAs outperform existing serologic and molecular biomarkers and how they are stabilized in circulation remains unknown.

Methods: We evaluated sera from patients with Nasopharyngeal carcinoma (NPC) vs. healthy controls collected from Indonesia (n = 72) for EBV-encoded microRNAs using stem-loop RT-PCR. In parallel we measured EBV-DNA load and anti-EBV IgA levels. Analysis of EBV microRNAs was also performed in independent patient population collected from Italy. Sera from patients with non-NPCs were included in the analysis as additional controls. Biological relevance of circulating EBV-encoded BARTs explored by size-exclusion chromatography (SEC) in serum fractions of patients with NPCs.

Results: We measured elevated levels of viral miR-BART7, miR-BART9 and miR-BART13 in sera, nasopharyngeal brush and tumor biopsy of groups of Indonesian patients with NPCs relative to healthy controls suggesting tumor relatedness and protection from RNase activity. A multivariable logistic regression and receiver operating characteristic (ROC) analysis showed that a combination of circulating cell-free EBV miR-BART7 and miR-BART13 differentiates patients with NPCs from healthy controls with an area under the curve (AUC) of 0.93. The performance of these EBV 2-miR-BARTs in an independent NPC population from Italy reached an AUC of 0.88. Strikingly, comparing NPC with non-NPC tumor controls and individuals with asymptomatic EBV infections (n = 70), serum miR-BART13 alone reached an AUC of 0.96, outperforming EBV serology and whole blood EBV-DNA levels that were analysed in parallel. Circulating EBV miR-BART13 and endogenous miR-155 are strongly associated with circulating extracellular vesicles in sera fractions of patients with NPCs suggesting that these are derived from living tumor cells.

Conclusions: Collectively, we conclude that circulating EBV-encoded BART microRNAs represent promising targets to diagnose patients with NPCs. Viral miR-BART13 is secreted from living EBV-infected NPC tumor cells and is stabilized in patient blood through its association with extracellular vesicles. A combined method detecting circulating cell-free EBV-DNA fragments and EBV-microRNAs simultaneously merits further study to serve an optimal strategy for non-invasive early detection of NPC and monitoring response to therapy.

Translational relevance

Analysis of tumor-derived EBV-DNA from cell-free fractions, namely plasma or serum is useful for screening patients at risk of NPC. One possible drawback of measuring circulating cell-free DNA fragments released by tumor cells upon apoptosis is that these may not always represent live tumor and could theoretically detect asymptomatic infections. Here the authors demonstrate comprehensive multi-analysis of EBV biomarkers using multiple specimens collected from patients with NPCs. Active secretion of the EBV-encoded miR-BART13 via extracellular vesicles in sera of patients with NPCs that have shown undetectable cell-free EBV-DNA or inconclusive EBV IgA in some indicating that EBV miR-BART13 or in combination with miR-BART7, strongly differentiates patients with NPCs from healthy donors and disease controls, outperforming EBV IgA serology and EBV-DNA detection in whole blood. Clinical potential of combined method detecting cell-free circulating EBV-DNA and EBV microRNAs as a non-invasive tool for NPC diagnosis assessment and real-time treatment monitoring warrants further study.

Introduction

NPC prevails in southern China, Southeast Asia including Indonesia, North Africa, the Middle East, and the Arctic[1]. Although NPC is rare in most other parts of the world, NPC incidence is increasing in low-risk populations such as Europe and USA due to worldwide migration[2,3]. Most patients with NPCs present in the clinic with advanced stages. Given the high risk of NPC in endemic regions and among certain family members[4], and the high cure-rate for early stage of disease[1], early detection of *NPC* using a non-invasive method is considered essential to reduce mortality burden for NPC in the high-incidence areas.

EBV is a primary etiologic agent in the pathogenesis of undifferentiated NPC[1,5,6]. Abnormal anti-EBV antibody profiles, increased circulating EBV-DNA levels, and a distinct EBV gene expression profile in tumor cells illustrate the active role of EBV in the tumorigenesis process[5-8]. Several blood-based EBV-assays have been accepted as circulating biomarkers for NPC, such as semi-quantitative ELISA for IgA antibodies against EBV-specific peptides and quantitative real-time PCR-based measurement of cell-free EBV-DNA[7-12].

Majority of individuals at high risk of NPC have elevated antibody titers to defined EBV antigens (EBNA1, EA, VCA) that persist asymptomatically up to ten years prior to

diagnosis[4,7,8,12-14]. Although these EBV markers proved to be sensitive for population screening and detecting incident NPC, ELISA-based assays for EBV serology do not meet the desired specificity for NPC diagnostic assessment[4]. Since EBV antibody levels are dynamic, the current frequency of NPC screening is usually adapted to the individual through antibody surveillance[7]. Oppositely, EBV IgA serology alone may lead to overdiagnosis of NPC and extra costs associated with additional clinical work-up in endemic countries.

Quantitative measurement of circulating cell-free viral load in plasma or serum by real-time PCR revealed higher specificity than serological test[1,8,10]. Previous studies done by us and others indicated that measuring cell-free and cell-associated EBV-DNA load in whole blood resulted a large variation of DNA copy numbers in patients with advanced stage of NPCs that includes undetectable viral load. Taken together, determination of the viral load in plasma had the highest specificity whereas assessment of EBV-DNA in whole blood was the least predictive of EBV status[8,16,17].

Measurement of plasma cell-free EBV-DNA load has been widely recognised as tumor marker for prognostic assessment of patients with NPCs undergoing radiotherapy and prediction of recurrent disease rather than for early detection of NPC[8,10,15,16]. This circulating EBV biomarker however does not reflect intact tumor cells[8,10,17]. In fact, viral infection is never cleared nor is EBV eliminated from the body. This suggests that certain viral molecules might prevent effective elimination of EBV-infected cells in patients with advanced NPCs by chemo or radiation treatments.

A non-invasive circulating biomarker that has high diagnostic accuracy (such as sensitivity, specificity, predictive values or AUC) is greatly desired for identifying high-risk subjects prior to the onset of clinical signs of NPC[18.19]. Ideally, such a marker would provide tumor-specific information to warrant further confirmation by nasopharyngeal brush sampling or tumor biopsy.

Circulating microRNAs (miRNAs) provide insight into both the biology and clinical behavior of disease including cancer[20-22]. EBV miRNAs play an important role in EBV transformation and NPC pathogenesis targeting anti-growth and apoptotic pathways[5,6]. Although a clear consensus has not yet emerged, several studies suggested that EBV miRNAs present biomarkers for NPC detection and treatment monitoring[19,23-26]. Defining circulating viral miRNA markers for NPC diagnosis assessment that directly reflect underlying tumor activity would have important clinical implications on early detection of NPC.

Here, we showed EBV miRNAs were being released by EBV-positive cells, tissues and found in a cell-free circulating form in serum of patients with NPCs. Although they are expressed differentially in different sample types, the expression levels of EBV miR-BART7, miR-BART9 and miR-BART13 were significantly higher in patients with NPCs compared with healthy individuals.

Diagnostic performance of circulating EBV miR-BART7 and miR-BART13 outperforms whole blood EBV-DNA and EBV IgA serology. Viral miR-BART13 is secreted from living EBV-infected NPC tumor cells and is stabilized in patient blood through its association with extracellular vesicles (EVs). Our data suggests EBV-miRNA based liquid biopsy approaches for NPC detection and diagnosis without the need for invasive and painful tissue biopsy. A combination of EBV-DNA and EBV miR-BART13 would be an ideal option to confirm inconclusive EBV IgA for early detection of NPC and to effectively measure treatment outcomes.

Material and methods

Patient selection and biological sample collection

Supplementary Fig. S1 shows the schematic flow chart of retrospective NPC circulating biomarker study. We used archived pretreatment samples from patients with NPCs from Indonesia (endemic) and Italy (non-endemic) including regional controls as described in recent publications[11,16,17,27]. We selected 56 patients with NPCs collected at the Dr. Cipto Mangunkusumo Hospital (Jakarta, Indonesia) with available frozen biopsy or nasopharyngeal brushing, serum and whole blood samples at diagnosis (Table 1). All patients were clinically staged according to the UICC/AJCC guidelines (supplementary Table S1). EBV status of tumor biopsies was assessed by EBER in situ hybridization[11].

Plasma samples from 14 histologically confirmed NPC collected at the National Cancer Institute, Centro di Riferimento Oncologico (Aviano, Italy) were used as independent cohort[27]. Healthy sera (n = 33) and plasma (n = 14) samples from Indonesia, Italy and Netherlands were tested as controls for miRNA and DNA analysis. In addition, a total of 31 pre-treatment sera from patients with various non-NPC diseases (Table 1) referred to VU Medical Center in Amsterdam, the Netherlands and Dr. Cipto Mangunkusumo Hospital, Indonesia[17] enrolled during the same period as patients with NPCs were tested for miRNA and anti-EBV IgA serology. All patients signed for informed consent and the study was approved by the medical ethical committees of the participating hospitals.

Table 1. Clinical characteristics of samples collected in this study

	Serum						Plasma		Tumor	
	Technical evaluation		Biomarkers				External group		validation	
	Non-malignant (n = 19)	Malignant (n = 17)	Healthy (n = 33)	NPC (n = 39)	EBV-associated diseases, except NPC (n = 7)	Non-NPC malignancies (n = 24)	Non-malignant (n = 14)	Malignant (n = 14)	Non-malignant (n = 22)	Malignant (n = 40)
Mean age (range)	35 (19-63)	41 (23-60)	36 (17-56)	40 (14-60)	36 (14-54)	61 (39-85)	30 (22-62)	44 (30-70)	23 (4-61)	40 (11-60)
Sex (Male/Female)	9/10	14/3	18/17	28/11	3/4	18/6	1/13	13/1	12/10	26/14
Ethnicity										
Asian	16	17	29	39	2	3	2		11	40
Caucasian	3		6		5	21	12	14	11	
Healthy donors	17		33				14		9	
Disease types										
Tonsilities									11	
Infectious mononucleosis	2				2					
Chronic active EBV					1					
Adenoid hyperplasia									2	
Tounge carcinoma						1				
Squamous cell carcinoma						2				
PTLD (lung, bone marrow transplantation)					2					
Non-hodgkin lymphoma					1					
NK/T cell lymphoma					1					
Non-NPC head and neck tumors						21				
Nasopharyngeal carcinoma		17		39				14		40

NPC, nasopharyngeal carcinoma; IM, infectious mononucleosis; CAEBV, chronic active EBV; NK/T-cell, Natural Killer T cell; PTLD, Post-transplant lymphoproliferative disease.

Cell lines

EBV-positive NPC cell line C666-1 and EBV-negative cell lines (NPC-derived HONE1, Burkitt lymphoma-derived BJAB) were cultured as previously described[28,29]. Upon confluence, media were removed, cells were harvested and RNAs were extracted for intracellular miRNA profiling[28,29]. The supernatants were collected and centrifuged at 500 × *g* for 10 min. To remove remaining cells and re-centrifuged at 2,000 × *g* for 15 min to eliminate cell debris[30]. Precleared supernatants were further analysed by size-exclusion chromatography (SEC).

Sample processing and nucleic acid purification

Serum, plasma, whole blood, nasopharyngeal brushing and frozen tumor biopsy samples were collected and processed for DNA and RNA isolation according to published protocols[11,17,29]. EVs were isolated from serum by SEC Sepharose CL-2B column (30 mL, GE Healthcare) as described[31,32]. Trizol LS (Invitrogen) was used to isolate total RNAs from 250 µL total serum or plasma according to the manufacturer's instruction. Glycogen (Invitrogen) was added to increase the RNA yield[11].

TRIzol (Invitrogen) was added to a. tumor cells, b. tumor biopsy, c. 250 µL of pooled SEC fraction 9 and 10 (EV fractions) and d. 250 µL of pooled fraction 20 and 21 (HDL/protein fractions). The total extracellular RNAs was precipitated and pooled from four times TRIzol-based RNA purification of EV and HDL/protein fractions. The rest of each fraction was stored at −80^0 C for subsequent western blot and transmission electron microscopy (TEM) as previously described[31,32]. DNA and RNA concentration and quality were assessed by NanoDrop ND-100[11,17,29]. RNA was stored at −80^0 C until further use.

RNA profiling of intra and extracellular miRNAs

We sequenced and profiled intra and extracelullar viral and human small non-coding RNAs of EBV-positive and EBV-negatives cell lines as mentioned above with the purpose of identifying tumor specific EBV miRNAs. Detail protocols were described in previous our published articles[29,32,33].

Molecular analysis

Viral and endogenous small noncoding RNA profiling was performed on 5 µL (250 to 350 ng RNA) total RNA extracted from cells, frozen biopsies, nasopharyngeal brush, serum and plasma samples with labeled primers (Life Technologies). All necessary reagents for noncoding small RNA-specific cDNA synthesis prior to multiplex stem-loop RT-PCR followed by real time PCR with TaqMan probes (Applied Biosystems AB7500) have been previously detailed[29,30]. Two small non-coding RNAs (viral EBER1 and host vault RNAs) were analysed by LightCycler stem-loop RT-PCR (Roche, LC480) as previously

published[33]. EBV-positive (C666-1) and and EBV-negative (HONE1) cells were used as calibrators. A true amplification curve with raw cycle threshold (Ct) mean value <40 (Ct SD <0.3) and was considered reliable in Taqman and LightCycler systems.

We used vault RNA and miRNA16-5p as endogenous controls[34,35]. A Ct value of 40 (Ct SD >0.3) for vault RNA and a Ct value >30 (Ct SD >0.3) for miRNA16-5p was considered as adequate levels for defining RNA quantity[32]. This way we were able to exclude possible false negatives in serum or plasma samples. Relative quantification of miRNAs was calculated using the $2^{-\Delta\Delta Ct}$ approach[34]. Normalization was performed with vault RNA[32,33]. EBV-DNA load was measured using protocol as published before[9,16,17]. All experiments were run as independent duplicates. Primers and probes used in this study are listed in supplementary Table S2.

EBV EBNA1 and VCAp18 antibody assays

IgA antibody levels against the EBV EBNA1 and VCAp18 proteins in serum samples from patients with NPCs, control patients and healthy controls (Table 1) was measured according to previously published protocols[4,12,13].

Clinical cut-off points and statistical methods

Graphical analysis was plotted using Prism 6.07 software (GraphPad) and statistical evaluations were executed by using SPSS 22 and R software (version 3.2.5). All tests were two-sided and considered statistically significant at the 0.05 level. The histogram and normality plots were applied to determine whether data followed a normal distribution. In miRNA analysis, $2^{(-\Delta\Delta Ct)}$ from each miRNA were square-root transformed, standardized and then evaluated by the non-parametric Rank Products test using the R statistics. In this study, serum EBV miRNA was presented in log2 transformed expression value, EBV-DNA load was presented as copies per ml whole blood or copies per biopsy/brush and anti-EBV antibody level was presented in mean per COV[4,17]. All values in the text and figures represent the mean or mean with SD.

The Receiver Operating Characteristic (ROC) analysis was performed to evaluate the diagnostic performance of *each* circulating EBV miRNA in differentiating patients with NPCs (n = 39) from the healthy control subjects (n = 33) and patient with other diseases (n = 31)[35]. By using these optimal cutpoints, sensitivity, specificity, positive and negative predictive values (PPV and NPV) of each individual circulating EBV marker in distinguishing NPC from each control group were calculated[35]. By analyzing serum associated EBV miRNA levels in patients with NPCs and healthy subjects, a diagnostic miRNA signature for NPC was built using logistic regression model[36]. Spearman correlation test was used to examine the relation between expression level of EBV-

encoded miRNA in the circulation and EBV-DNA load in primary tumor by using the log2 scale of quantitative EBV-DNA level and the Ct value of each miRNA. Asterisks indicate statistical significance – * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$).

Results

We previously performed comprehensive EBV miRNA profiling studies of EBV-associated tumors and cell lines showing that EBV-miRNA expression patterns can define tumor type[28,29]. Here we wished to determine whether EBV miRNAs released by NPC tumors into circulation are useful biomarkers for non-invasive NPC diagnosis. To this end we sequenced NPC cell line carrying natural EBV genome, C666.1, and its cell-free supernatant and we identified four tumor-derived EBV miR-BARTs (6-3p, 7-3p, 10-3p and 17-5p) as shown in supplementary Fig. S2A-B.

According sequencing and previous results[28-30], we further analysis the ten abundantly expressed viral non-coding small RNA candidates and several endogenous miRNAs[19,37] to a semi-quantitative real-time stem-loop reverse-transcription PCR (Taqman RT-qPCR) using EBV-positive and EBV negative cells and their cell-free supernatants fractionated by SEC[30,31,32,34] (supplementary Fig. S2C-E).

In vitro profiling revealed abundant expression of four EBV miR-BARTs (7-3p, 9, 10, 13) in EBV-infected NPC cells (supplementary Fig. S2C) that were also included in both the extracellular vesicle and protein/HDL fractions (supplementary Fig. S2D). The intracellular levels of miRNA BART6 and small non-coding EBER1 were much higher than the secreted levels (Figs. S2C, S2D), suggesting this miRNA may not be secreted in circulation. Interestingly, miRNA profiling of the SEC fractions revealed the near absence of miR-BART17-3p secreted by EBV-associated tumor cells, whereas viral miR-BART7-5p, miR-17-5p, and miR-BHRF1-1 were expressed at low levels in both intra and extracelullar compartments. Control cell lines lacking the EBV genome confirmed true negative EBV signals (Figs. S2C, S2E).

Endogenous human miRNAs were measured at equal expression levels in EBV-positive and negative cell lines, except for miR-155, which was decreased in C666.1 cells. The cellular miR-16 (data not shown) and small non-coding vault RNA were stably expressed among tumor cells (supplementary Fig. S2C), fractionated cell-free supernatant (supplementary Fig. S2D) and among various types of clinical specimens (Fig. 1A). Therefore, both are considered as appropriate internal small RNA controls.

This initial assessment in clinical specimens (data not shown) lead to the selection of EBV miR-BART7, miR-BART9, miR-BART13 and endogenous miR-155 as a quality control for further analysis[23,26,32,38-40]. To assess whether these three EBV miRNAs were derived primarily from tumors, we evaluated their expression levels individually in sera (n = 39) of patients with NPCs, and matched nasopharyngeal NPC brushings (mucosal scrapes of tumor surface, n = 20) with direct frozen NPC biopsies (n = 20) collected from the same patients. Sera from healthy individuals (n = 33), nasopharyngeal brushings obtained from a group of healthy EBV-carriers (n = 11) and hypertrophic tonsil tissues (n = 11) served as controls.

We measured elevated levels of EBV 3-miR-BARTs in sera, brush and biopsy specimens revealing a significant difference between patients with NPC and their controls ($P < 0.0001$, Figs. 1B-D). The relative expression levels of EBV miR-BARTs in the nasopharyngeal brushings and biopsies of patients with NPC was similar, suggesting that EBV miR-BARTs in nasopharyngeal brushings represent NPC tumor presence.

Circulating EBV miRNAs discriminate NPC patients from control groups

Given the differential intra and extracellular EBV miRNA expression, our next question was whether circulating cell-free EBV miRNAs could be useful for detecting NPC. We analysed serum specimens obtained from 39 patients with NPCs and controls that contained healthy (n = 33) and patients with non-NPCs (n = 31) for the expression of circulating three EBV miRNAs. The non-NPC population had a proportion (n = 7) of EBV-associated diseases (Table 1). We used miR-155 for sample-to-sample comparison[37].
We noted that the average Ct value for vault RNA is equal to EBV miRNAs, in contrast to miR-16, thus serving as a suitable reference marker for relative expression levels of EBV miRNAs (Fig. 1A). However, the expression level of each EBV miR-BART candidates remains significantly different from control groups with or without normalization (data not shown).

We found that the relative expression levels of each circulating cell-free EBV miR-BART7, miR-BART9, and miR-BART13 discriminate the patients with NPCs from healthy subjects ($P < 0.0001$) and non-NPC patients ($P < 0.0001$; Fig. 2A). This suggests that circulating viral miRNAs strongly associated with the presence of NPC tumors. In contrast, miR-155 levels are similar between groups proposing that miR-155 is not indicative for NPC status, but possibly reflects more general lymphoid inflammation, thus it was excluded from further analysis (Fig. 2A). While healthy subjects and non-NPC controls have a detectable but low background of EBV miR-BART7 and miR-BART9, circulating miR-BART13 was barely detectable in sera from healthy EBV-carrying individuals (Fig. 2A).

When comparing patients with EBV-associated non-NPCs (i.e. infectious mononucleosis, chronic active EBV infection and natural killer/T cell lymphoma) as a separate group vs.

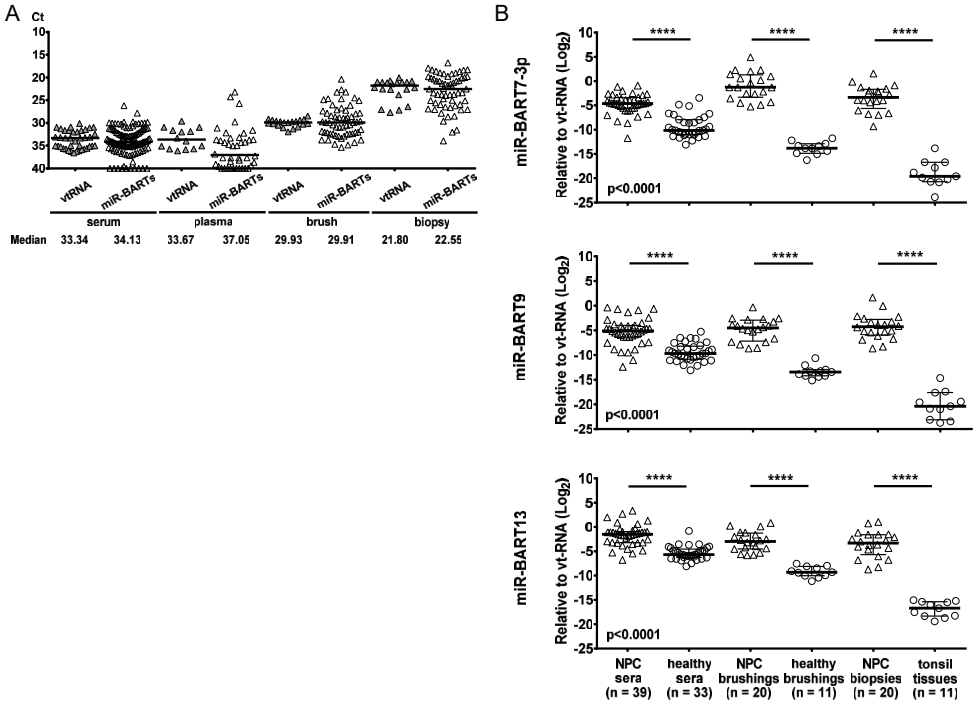

Figure 1. EBV miR-BARTs (7, 9, 13) are present in tumor biopsies, nasopharyngeal brushes, plasma and sera of patients with NPCs. A, stable expression of non-coding vault RNA presents the levels of EBV miRNAs in vivo and serves as a normalizer for EBV miR-BART biomarkers. In NPC sera, brush and biopsy, median Ct value of vault RNA (33.34, 29.93, 21.80) was similar to median Ct value of combined three EBV miR-BARTs (34.13, 29.91, 22.55). B, Expression levels of three EBV miR-BARTs (7, 9, 13) is higher in all specimens collected from patients with NPCs than control healthy subjects.

patients with NPCs, the level of each circulating EBV miR-BART remained statistically significant higher ($P = 0.01$ for miR-BART7 and miR-BART9, $P < 0.0001$ for miR-BART13, supplementary Fig. S3). Our data indicate that these three EBV miR-BARTs are secreted and detectable in the circulation of patients with EBV-related diseases however their levels are significantly more elevated in patients with NPCs (Fig. 2A).

Circulating EBV miR-BART13 outperforms EBV-DNA and EBV IgA serology

We next calculated the diagnostic potential of each EBV markers using receiver operator characteristics (ROC) in sera of patients with NPCs (n = 39) vs. healthy (n = 33) or patients with non-NPCs (n = 31)[35]. As compared to healthy donors, the ROC curve of individual viral miRNAs revealed AUC's of 0.90, 0.87, and 0.91 for miR-BART7, miR-BART9, and miR-BART13, respectively suggesting the potential of EBV miR-BART13 as NPC signature (Fig. 2B). Compared to EBV IgA serology (data not shown), the least predictor for NPC was EBV-DNA whole blood with an AUC of 0.55 (Fig. 2B).

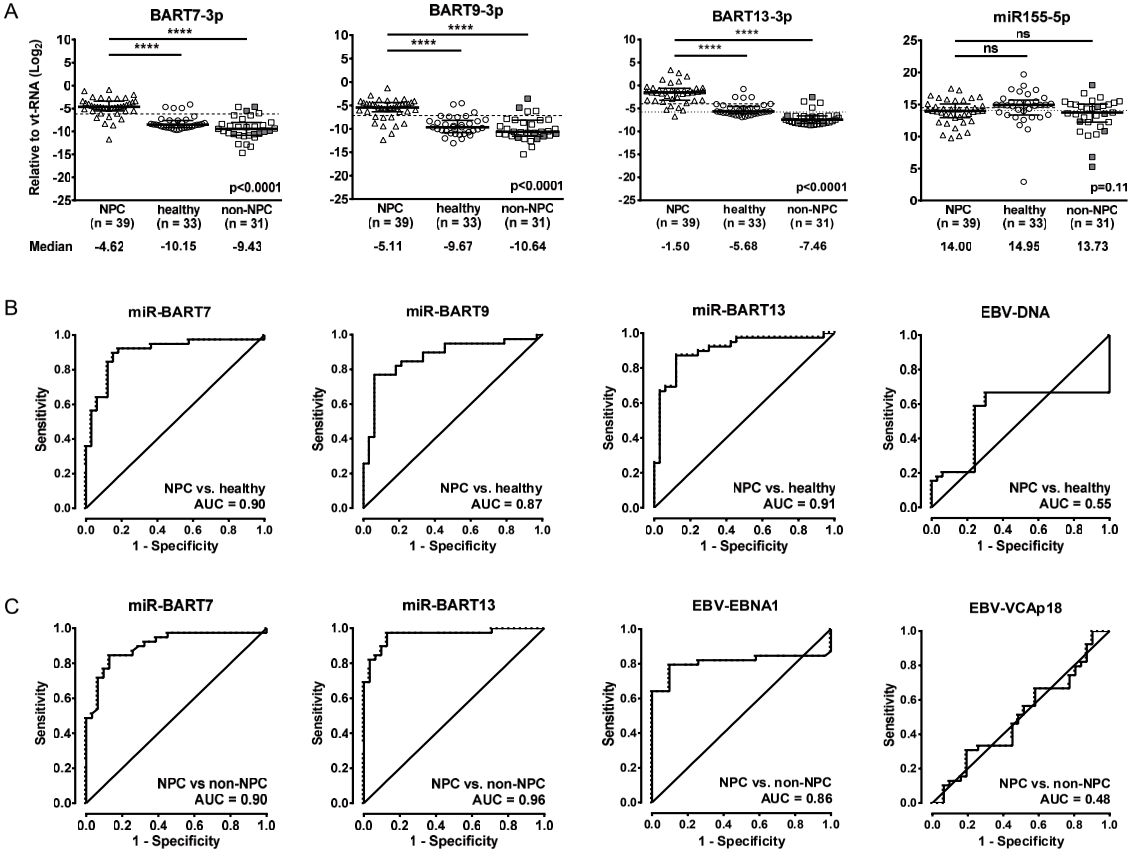

Figure 2. Serum levels of circulating EBV miRNAs are elevated in patients with NPCs and differentiate them from control groups. Serum levels of circulating EBV miRNAs are elevated in patients with NPCs. a). Relative expression of each circulating ebv-miR-BARTs (7, 9, 13) was significantly higher in NPC compared to two-control groups. No significant difference between NPC patients and controls for circulating miR-155. Significant differences were analyzed by Mann-Whitney U tests, and the p-values are indicated above the plots (**** $P < 0.0001$). Thick black line represents mean, dot line represents cut-off point. ROC curves for serum-based miRNAs compared to b) healthy subjects and c). patients with various type of diseases except NPC. The AUC value of each EBV miR-BARTs and EBV-DNA for distinguishing patients with NPCs from healthy subjects was 0.90, 0.87, 0.91, and 0.55. To discriminate NPC from non-NPC patients the AUCs of miR-BART7 and miR-BART13 (0.90 and 0.96) were higher than the AUC's of IgA-EBNA1 and IgA-VCA (0.86, 0.48), respectively.

Most interestingly, the AUC values of viral miR-BARTs are equally high when patients with NPCs are compared to patients with non-NPCs (miR-BART7-3p 0.90 and miR-BART13 0.96, Fig. 2C). Next to EBV miR-BARTs, IgA anti-EBNA1 ELISA yielded an AUC of 0.86 (sensitivity, 0.92 and specificity, 0.71) which was superior to IgA-VCAp18 (AUC, 0.48; sensitivity, 0.36 and specificity, 0.74, respectively) in discriminating NPC. These results are depicted in Table 2.

Table 2. Circulating cell-free EBV miR-BART13 outperforms EBV-DNA and EBV-IgA for detection of NPC patients

Parameters	AUC	Sensitivity	Specificity	PPV	NPV
miR-BART7[a]	0.903	0.846	0.909	0.917	0.833
miR-BART9[a]	0.873	0.744	0.970	0.967	0.762
miR-BART13 [a]	**0.906**	**0.821**	**0.970**	**0.970**	**0.821**
EBV-DNA load [a]	0.548	0.667	0.697	0.722	0.639
miR-BART7[b]	0.902	0.846	0.871	0.892	0.818
miR-BART13 [b]	**0.961**	**0.974**	**0.871**	**0.904**	**0.964**
IgA-EBNA1 [b]	0.864	0.923	0.710	0.800	0.880
IgA-VCAp18 [b]	0.476	0.359	0.742	0.636	0.479

a. NPC vs. healthy EBV-carriers
b. NPC vs. non-NPC, with EBV-associated diseases in some

Taken together, our data shows that circulating EBV miR-BART13 is the most potential biomarker to distuingish patients with NPCs from healthy EBV-carriers and patients with other diseases including those that have been reported to be EBV-associated. The diagnostic parameters of each individual EBV biomarker is presented in Table 2.

EBV miR-BART13 alone or in combination with miR-BART7 serves as NPC signature

Next, we used the information of ROC analysis to determine the optimal cut-off point for each of the three circulating cell-free EBV miRNAs to reach maximal sensitivity and specificity as identified by R3.2.5 software. To discriminate patients with NPCs from healthy individuals, the optimal cut-off points for circulating serum EBV miR-BART7, miR-BART9 and miR-BART13 were 0.50, 0.66, and 0.53, which allowed the relative expression values to be categorized as positive or negative for NPC[35]. The analysis demonstrated that the three EBV miRNAs provide high sensitivity and specificity (miR-BART7, miR-BART9, miR-BART13: 0.85 and 0.91; 0.74 and 0.97; 0.82 and 0.97, respectively) with circulating EBV miR-BART13 being the most strongly NPC-associated miRNA (Table 2). Logistic regression analysis of NPC vs. healthy indicated that measurement of a diagnostic

Table 3. Circulating EBV 2-miRNA signature defines NPC cases

Parameters	AUC	Sensitivity	Specificity	PPV	NPV
miR-BART7+miR-BART13[a]	0.930	0.872	0.909	0.919	0.857
miR-BART7+miR-BART13[b]	0.883	0.857	0.857	0.866	0.866

a. Sera of patients with NPCs and healthy subjects collected from Indonesia (n = 72)
b. Plasma of patients with NPCs and healthy subjects collected from Italy and Netherlands (n = 28)

EBV 2-miRNA signature (miR-BART7 and miR-BART13) provides the best model (AUC, 0.93) to predict NPC with 0.87 sensitivity, 0.91 specificity, 0.92 PPV, and 0.86 NPV, Table 3).

Circulating EBV 2-miRNA signature defines NPC cases in independent NPC population

We evaluate the NPC diagnostic signature in NPC plasma, an independent sample population collecting from Italy (non-endemic). Consistent with our data in NPC sera from Indonesia (endemic), a combination of EBV miR-BART7 and miR-BART13 reached a high AUC of 0.88 with both sensitivity, and specificity of 0.86 (Table 3). These data show that EBV 2-miRNA signature may be a potential non-invasive biomarker for NPC in both endemic and non-endemic regions. However, it is possible that the number of Italian collected in this study was very small to sufficiently evaluate these two EBV miRNAs as representative of patients with NPCs cohort from non-endemic region.

Circulating cell-free EBV miR-BART13 is enriched in EVs from patients with NPCs

Circulating EBV-DNA likely represents pieces of viral genome that are released by dying cells. We hypothesize that at least a proportion of circulating EBV miR-BARTs in sera of patients with NPCs sera might be actively secreted by living tumor cells, possibly via exosomes[41-43]. To test this possibility, we performed SEC-based fractionation into EVs and protein/HDL complexes[31,32] using sera (n = 15) of patients with NPCs (supplementary Table S1) followed by stem-loop RT and Taqman small/miRNA assays. As controls, we also performed SEC analysis in sera (n = 9) from healthy subjects. A representative image confirmed the successful isolation and purification of serum EVs in fractions 9 and 10 (Fig. 3A) while protein/HDL aggregates are present in fractions 20 and 21 and no EVs could be observed (Fig. 3B).

While EBV miR-BART9 was undetectable and EBV miR-BART7 was detected in both EV and protein/HDL fractions (data not shown), EBV miR-BART13 (Fig. 3C) was significantly enriched in the EVs from patients with NPCs (n = 15) compared to healthy controls (P = 0.008) whereas EV-associated endogenous miR-155 showed a similar distribution difference in NPC cases and controls (P = 0.355, Fig. 3D).

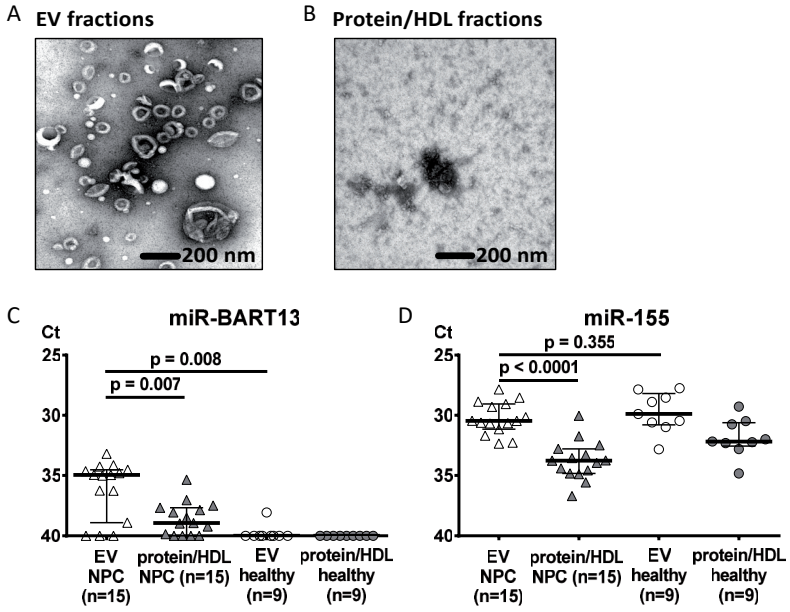

Figure 3. Circulating EBV miR-BART13 is enriched in circulating EVs from NPC patients. EM images of A, EVs and B, protein/HDL fraction of serum-based SEC. C, RT-PCR analysis of serum fractions indicated that EVC miR-BART13 was enriched in EV fractions ($P = 0.007$) of patients with NPCs ($P = 0.008$). D, Endogenous extracellular EV-enriched miR-155 ($P < 0.0001$) was not associated with NPC ($P = 0.36$). Thick black line represents mean, white filled circles and squares represents EVs, grey filled circles and squares represent protein/HDL complexes.

The detection and quantification of extracellular miRNAs in serum is equally aplicable to plasma (n = 4, supplementary Table S1). We found no difference in expression level of serum and plasma's EBV miR-BART13 in these archived materials (data not shown). These findings suggest that some viral non-coded small RNAs are sorted into the extracellular space in a non-selective manner. In addition, EBV miR-BART13 that actively secreted by living tumor cells may have functional activity in exosomes[43,44].

Evaluation of circulating EBV molecular markers

We then wondered whether circulating EBV miRNAs correlate with viral load in blood and whether the absence of viral miRNAs would reflect absent expression of circulating cell free EBV-DNA copies.

Analyzing the correlation of log2 ratio of EBV-DNA copies in whole blood and the levels of serum EBV miR-BARTs individually, we found that there was a significant positive correlation between high viral loads and high circulating each EBV miR-BART, as displayed in Fig. 4A (miR-BART7, miR-BART9, miR-BART13: $P = 0.0037$, $P = 0.0055$, P

< 0.0001 respectively). However, this was not seen for miR-155 (data not shown). We noted absence of only one or two individual EBV miR-BARTs in 3 out of 39 NPC cases, whereas most cases with absent EBV-DNA (n = 12) showed clearly detectable EBV miR-BART markers (data not shown).

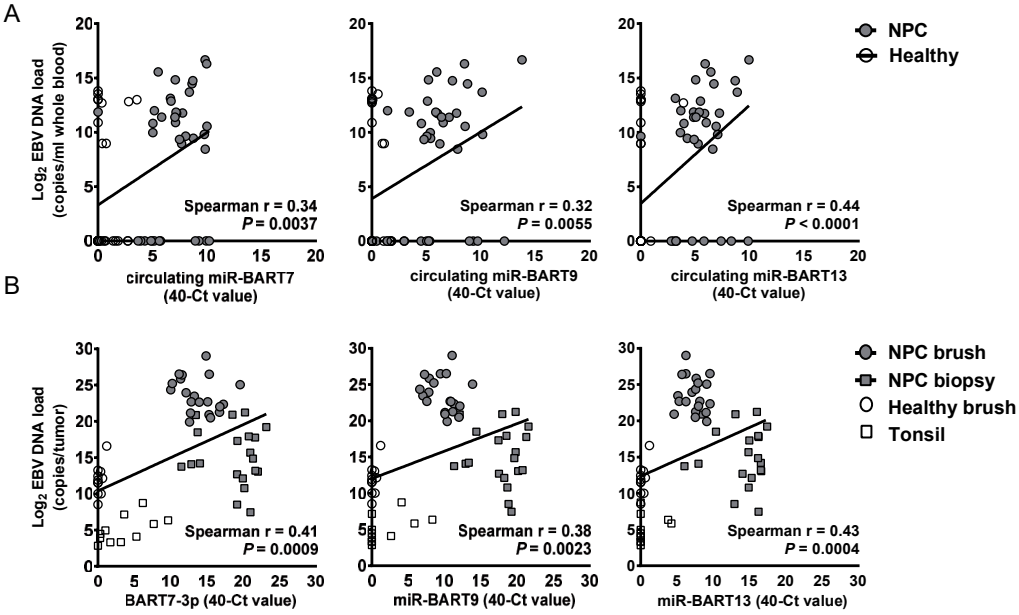

Figure 4. EBV-miRNA correlates with EBV-DNA load in circulation and tumor. A significant positive correlation was found between high expression levels of A, each circulating EBV miR-BARTs (7, 9, 13: $P = 0.0037$, $P = 0.0055$, $P < 0.0001$) and high log2 scale cell-free EBV-DNA copy number, as identified by Spearman correlation (7, 9, 13: r=0.34, 0.32, 0.44), and B, each intracellular EBV miRNA and high log2 scale tumor-associated EBV-DNA load (miR-BART7 r = 0.41, $P = 0.0009$; miR-BART9 r = 0.38, $P = 0.0023$, miR-BART13 r = 0.43, p=0.0004, respectively). Grey filled circles and squares represent NPC patients, white filled circles and squares represent healthy subjects.

Consistent with previous published findings the EBV-DNA level in nasopharyngeal brushings and tumor biopsies from patients with NPCs was very high compared to controls[9,11,17]. We also investigated a direct relationship between EBV-DNA load and EBV miRNA levels in NPC tissues as we observed in circulation. As expected, the analysis demonstrates a strong positive correlation between EBV-DNA load and the expression of each EBV miRNA as shown in Fig. 4B (miR-BART7, miR-BART9, and miR-BART13: $P = 0.0009$, $P = 0.0023$, $P = 0.0004$, respectively). In contrast, we identified a significant negative correlation between endogenous miR-155 and log2 ratio of EBV-DNA load in the primary tumors (data not shown). Together these results suggest that circulating EBV miRNAs are derived from EBV infected NPC tumor cells.

Discussion

For most cancers, early and specific markers are lacking. Hence, besides the continuous refinement of existing biomarkers, the search for novel, early disease predictors representing active tumor cells belongs to the current challenges in biomarker research[18]. The fundamental role of EBV miRNAs in the pathogenesis of EBV-associated diseases, their presence and stability in biological fluids has led to extensive investigation of EBV miRNAs as potential non-invasive biomarkers for NPC diagnosis and prognosis.

To date, non-invasive EBV IgA serology and molecular EBV-DNA tests confirmed by endoscopic assessment and pathological examination of tissue biopsies is the general approach for screening and diagnosis of NPC[1,4,7,13,14,47,48]. EBV-DNA measurement is also applied for NPC treatment monitoring[8,10,16,17]. In this study we evaluated the efficacy of circulating cell-free EBV miRNAs as biomarkers for NPC and compared their performance with existing non-invasive EBV-based assays including cell-free EBV-DNA load and EBV IgA serology[9,11-13,16,17].

Our data using Indonesian NPC cohort suggest that measuring EBV miR-BART13 levels (AUC of 0.91) alone already outperforms (Fig. 2, Table 2) the diagnostic performance of IgA-EBNA1 and IgA-VCAp18 (AUCs of 0.86 and 0.48) and EBV-DNA (AUC of 0.55). A multivariable logistic regression analysis showed that the EBV 2-miRNA signature (miR-BART7 and miR-BART13) can thus improve the diagnosis sensitivity and specificity for NPC when compared with a single non-invasive biomarker (AUC of 0.93, Table 3).

However, despite the indisputable progress in miRNA field, the diagnostic promise of circulating EBV miRNAs has remained a work in progress and it requires larger groups of patients to demonstrate their clinical utility. Commonly reported miRNAs as disease biomarkers are largely not disease-specific and the results are often contradicting in independent studies. Here, we analysed the EBV 2-miRNA signature in Italian NPC cohort resulting a consistent high AUC of 0.88 (Fig. 3B). The result of this research has suggested that alterations of EBV miR-BART13 alone or in combination with miR-BART7 may indeed be the best predictor of NPC.

Four retrospective studies are worth discussing and comparing to our analysis. Zhang et al.[23] and Chan et al.[38] reported that circulating EBV miRNAs, miR-BART7 and miR-BART13, might constitute useful new molecular biomarkers for diagnosing NPC[23,38] and predicting treatment efficacy[23]. Unlike us, biological relevance of EBV miRNAs and comparison with existing NPC markers were not explored in these two studies. Nevertheless, the stage distribution was similar to our analysis with a predominance of

patients with stages III and IV.

Other studies found that relative expression levels of BART17-5p differentially expressed plasma miRNAs, as identified by qPCR, could be helpful for detecting primary NPC in patients with advanced disease[24] and predicting survival in patients with NPC[25]. Both studies indicated that different protocols resulted in different positive cases. In agreement with Chan et al[25], circulating serum-miR-BART17 was barely detectable in our study. All these studies suggested that circulating miRNA might serve as new biomarkers for NPC. However, biological relevance of these dysregulated EBV miRNAs and comparison of EBV biomarkers using self-paired samples have never been explored.

While circulating secreted EBV miRNAs seem promising, samples from nasopharyngeal brushes are considered to strongly and directly reflect the local tumor load and disease process[11,48-50]. In agreement with previous reports, our results showed indeed that EBV-DNA and EBV miR-BARTs were both highly expressed in nasopharyngeal brushings and tumor biopsies[38,48-50]. Therefore, similar with others[25,38] we propose multi-sample EBV-based analysis to support clinical NPC treatment decisions as illustrated in Fig. 5.

As part of mass awareness and education programs across endemic regions, EBV serological marker, IgA-EBNA1, is useful for screening NPC-risk individuals[4,7,12-14]. PCR-based circulating cell-free EBV miR-BART13 alone or in combination with circulating cell-free miR-BART7 and EBV-DNA[16,17,25,38,47] will first be completed in blood samples of risk individuals with highly elevated IgA-EBNA1 antibody titers before performing invasive tumor biopsy. EBER RNA in situ hybridization (EBER-RISH) and pathological examination is performed for final diagnosis of positive NPC.

Although repeated brush sampling is technically feasible, this is an invasive and bothersome procedure[9,16,17] therefore we propose brush sampling as an option to follow up inconclusive circulating cell-free DNA and/or miRNA results thus rule out false negatives[25,38,46,47] (Fig. 5). Together this data supports the idea that measuring EBV-DNA or EBV miR-BARTs in nasopharyngeal brushes is a valid option for in situ NPC tumor confirmation after liquid biopsy (EBV IgA EBNA1, circulating cell-free EBV miR-BARTs and/or EBV-DNA) analysis as indicated in previous studies[11,17].

Could the circulating cell-free EBV miRNA approach be improved and do they have a biological role as we found previously?[39] EBV miR-BART13 in exosomes was recently found to have a possible role in an EBV-associated auto-immune disease[51]. We determined by SEC that at least two different populations of circulating EBV miRNAs exist, a small portion of circulating EBV miRNAs presumably released by tumor cells

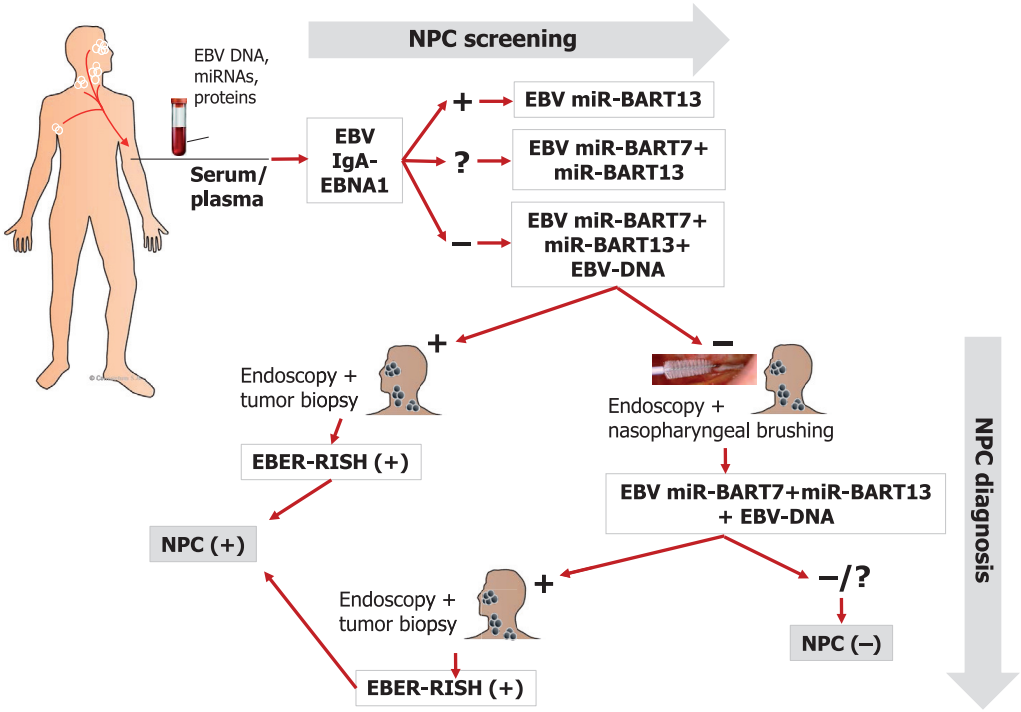

Figure 5. Proposed scheme of combined multiple EBV biomarkers for NPC screening and diagnosis

as free molecules and rapidly degraded by nucleases in blood while another large portion may originate from the living tumor cells as they are exported via active EVs and ribonucleoprotein complexes[21,24].

Selective packaging of cell free miRNAs into carriers is probably related to the specific biological functions of the secreted miRNAs[52]. Gourzones et al. reported that circulating miR-BART17-5p is co-purified with a protein-rich fraction but not with exosomes, which suggests the non-exosomal transport of miR-BART17-5p. In contrast, our findings of EBV miR-BART13 and endogenous miR-155 (Fig. 3) as strongly secreted and EV-associated miRNAs in patients with NPCs warrant further examination.

One limitation of this study is the relatively low numbers of early stage disease included in our cohorts precluding us to state with certainty that EBV miRNAs are superior over serology in detecting early stage disease. However since the sensitivity and specificity we measured by ELISA in our cohorts, correspond very well with published larger-scale studies[13], we feel it is safe to conclude that our comparative analysis warrants a large scale multi-center validation of circulating EBV-encoded miRNAs as a promising complementary or alternative to serology. If combined with circulating cell-free EBV-

DNA, it may even be the first-line test for NPC screening[38].

Next steps are standardization & harmonization efforts using common protocol to measure circulating cell free EBV-encoded DNAs and miRNAs that involve collaborations and consortia are important to minimise single-center bias in order to reach public acceptance of clinical utility of combined EBV non-invasive biomarkers for NPC.

Conclusions

EBV miRNAs can be generally linked to EBV-associated cancers as exemplified for NPC-predicted miR-BART13 because of its secretion from active EBV-infected NPC tumor cells. Based on our multivariable analysis, we conclude that the PCR-based EBV 2-miRNA signature (EBV miR-BART7 and miR-BART13) outperforms existing EBV-based assays and serves as the best non-invasive biomarkers for discriminating patients with NPCs from asymptomatic EBV-carriers and other diseases. This result warrants further evaluation of circulating EBV miRNAs as an alternative clinic-based screening method for identifying patients with NPC from patients with generic head and neck complaints. Evaluation in larger cohorts is required to establish clinical utility of EBV miRNAs and EBV-DNA as an optimal strategy for non-invasive early detection of NPC, monitoring NPC progression and predict therapeutic outcomes.

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Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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Supplementary

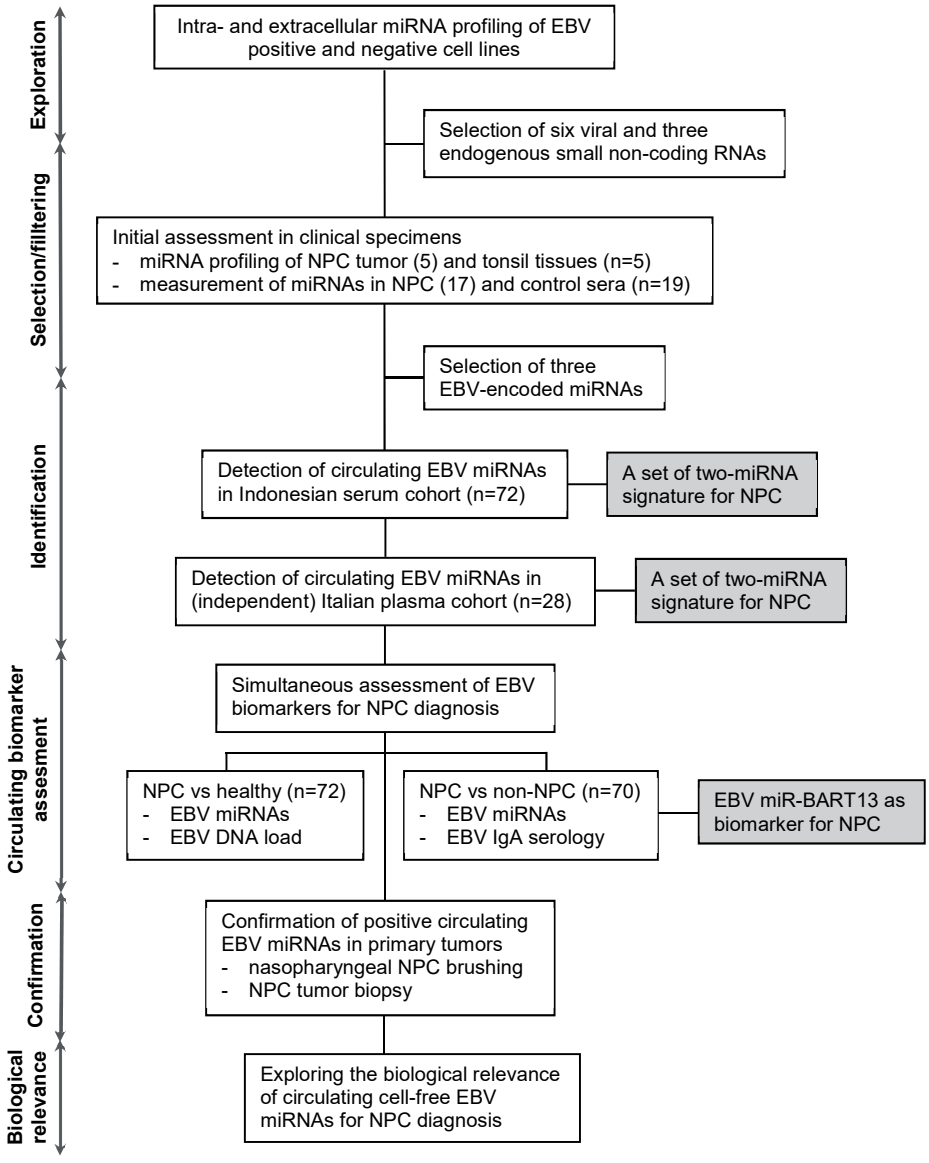

Figure S1. Workflow of NPC circulating biomarker study

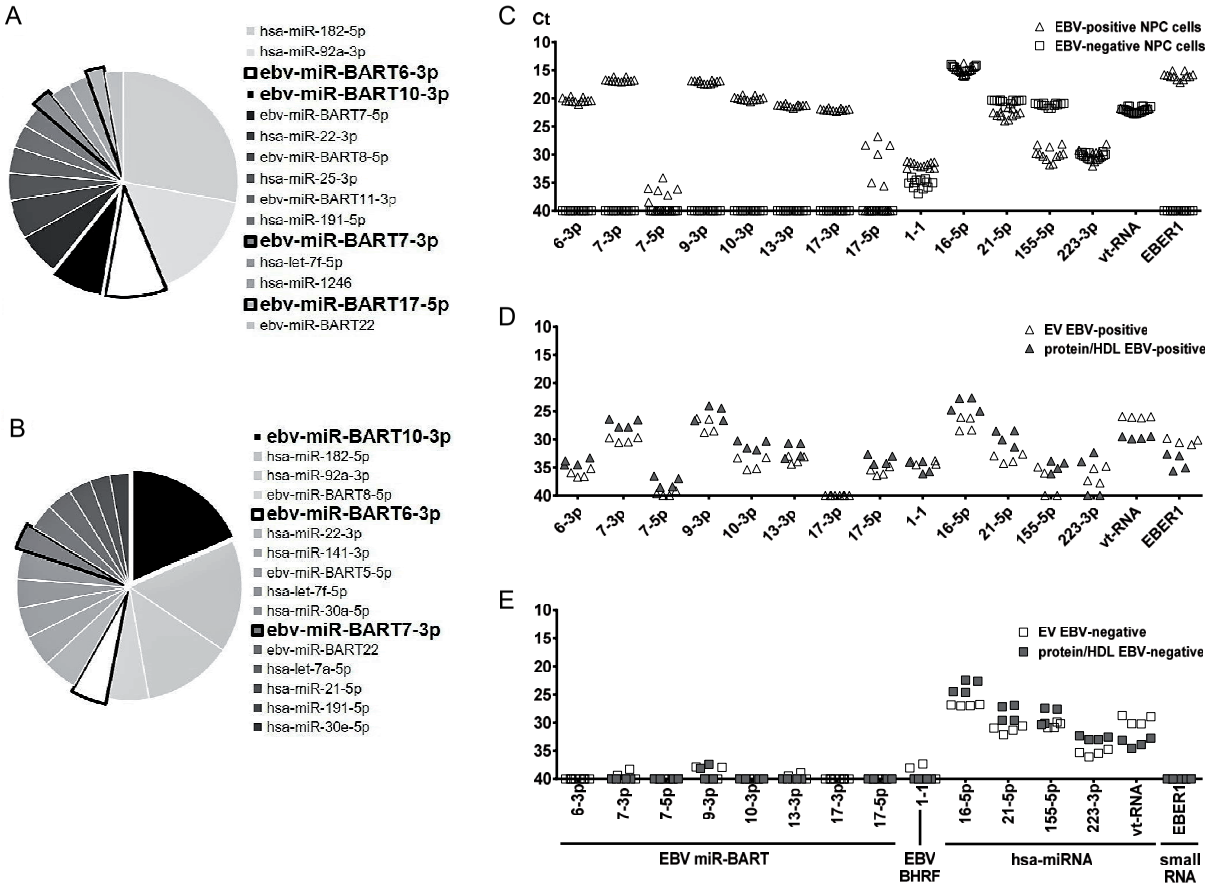

Figure S2. EBV miR-BARTs (7, 9, 10, and 13) are secreted via extracellular vesicles (EVs) and bound to protein complexes. Sequencing analysis of EBV-positive, C666.1 (A) and its cell-free supernatant (B). C, Intracellular EBV-encoded and endogenous miRNA profiling in EBV-positive (C666.1) and EBV-negative (HONE1) NPC cell lines.* D, Extracellular EV-associated and protein/HDL-associated EBV-encoded and endogenous miRNA profiling in EBV-positive C666.1 cell line. E, Extracellular EV-associated and protein/HDL-associated EBV-encoded and endogenous miRNA profiling in EBV-negative BJAB cell line. *EBER1, viral-small noncoding RNA; Vault RNA, host-small noncoding RNA.

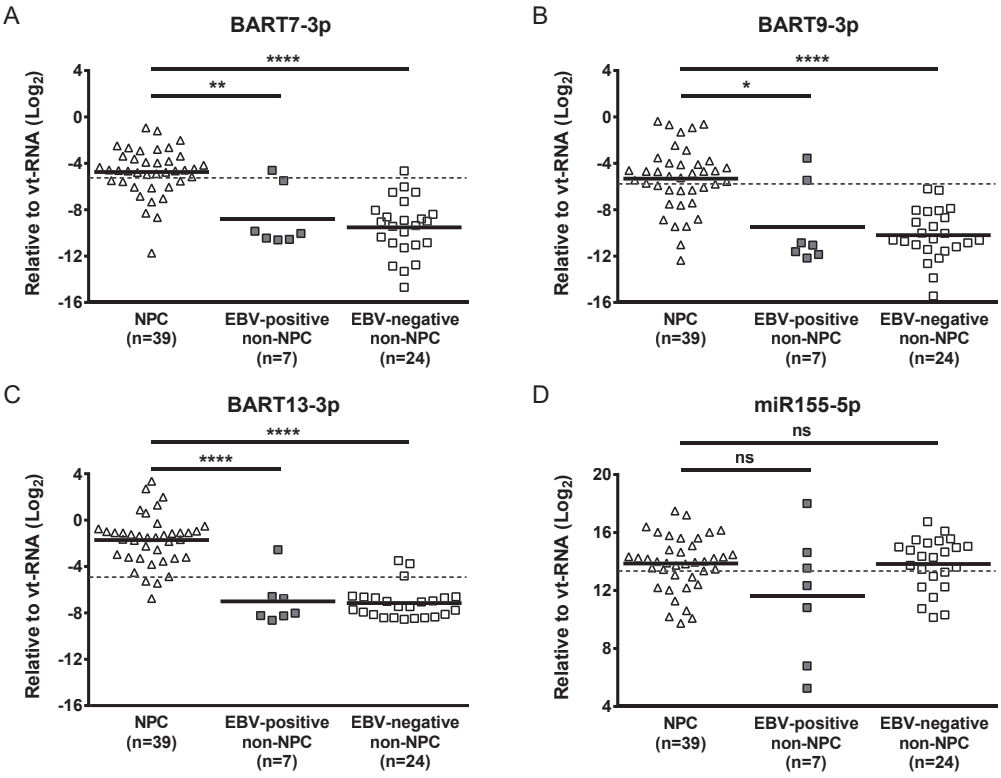

Figure S3. EBV miR-BARTs are highly specific for NPC. Relative expression of each circulating EBV miRNA – A, miR-BART7, B, MiR-BART9, C, miR-BART13 – was significantly higher in patients with NPCs compared to patients with EBV-positive non-NPCs and EBV-negative non NPC diseases, whereas D, Endogenous miR-155 showed no significant different between NPC and other two groups. Significant differences were analyzed by Mann-Whitney U tests, and the p-values are indicated above the plots (* $P < 0.05$, ** $P < 0.005$, **** $P < 0.0001$). Thick black line represents mean, dot line represents cut-off point.

Table S1. The tumor stage of NPC cohort

Tumor stage	Specimen						
	Blood (n=56)	Serum (n=56)	Serum fractions (n=15)	Plasma (n=14)	Plasma fractions (n=4)	Biopsy (n=20)	Brush (n=20)
Stage I	2 (3%)	2 (3%)		1 (7%)			
Stage II	10 (18%)	10 (18%)		6 (43%)	2 (50%)	3 (15%)	1 (5%)
Stage III	14 (25%)	14 (25%)	4 (27%)	5 (36%)	1 (25%)	3 (15%)	4 (20%)
Stage IV	30 (54%)	30 (54%)	11 (73%)	2 (14%)	1 (25%)	14 (70%)	15 (75%)

Table S2. Primers and probes used in this study

Name	Sequence	Ref
1. EBV EBNA1-DNA load (99 bp)		
Sequence	CCACAATGTCGTCTTACACCATTGAGTCGTCTCCCCTTTGGAATGGCCCCTG GACCCGGCCCACAACCTGGCCCGCTAAGGGAGTCCATTGTCTGTTAT	10,12
Forward	CCACAATGTCGTCTTACACC	
Reverse	ATAACAGACAATGGACTCCCT	
Probe	TCTCCCCTTTGGAATGGCCCCTG-FLUORESCEIN LCRED640-ACCCGGCCCACAACCTG	
2. β-globin DNA qPCR (196 bp)		
Sequence	GAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACA GACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAA GGTGAACGTGGATGAAGTTGG TGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTAC	53
Forward	GGAGAAGTCTGCCGTTACTGC	
Reverse	TTGGTCTCCTTAAACCTGTCTTGT	
Probe	CCAGGGCCTCACCACCAACTTC CCACGTTCACCTTGCCCCACAG	
3. Small non-coding RNA and microRNA stem-loop RT-PCR*		29,33
a. ebv-miR-BART6-3p		
Sequence	CGGGGAUCGGACUAGCCUUAGA	29
RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCTAAGGC	
Forward	ACACTCCAGCTGGGCGGGGATCGGACTAGC	
Probe	TTCAGTTGAGTCTAAGGC	
b. ebv-miR-BART7-3p		
Sequence	CAUCAUAGUCCAGUGUCCAGGG	29
RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCCTGGAC	
Forward	ACACTCCAGCTGGGCATCATAGTCCAGTGT	
Probe	TTCAGTTGAGCCCTGGAC	
c. ebv-miR-BART7-5p		
Sequence	CCUGGACCUUGACUAUGAAACA	29
RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTTTCAT	
Forward	ACACTCCAGCTGGGCCTGGACCTTGACTAT	
Probe	TTCAGTTGAGTGTTTCAT	
d. ebv-miR-BART9-3p		
sequence	UAACACUUCAUGGGUCCCGUAGU	26
RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACGGGA	
Forward	ACACTCCAGCTGGGTAACACTTCATGGGTC	
Probe	TTCAGTTGAGCTACGGGA	
e. ebv-miR-BART10-3p		
sequence	UACAUAACCAUGGAGUUGGCUGU	29
RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAGCCAA	
forward	ACACTCCAGCTGGGACATAACCATGGAGTT	
probe	TTCAGTTGAGACAGCCAA	
f. ebv-miR-BART13-3p		
sequence	UGUAACUUGCCAGGGACGGCUGA	29
RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAGCCGT	
forward	ACACTCCAGCTGGGTGTAACTTGCCAGGGA	
probe	TTCAGTTGAGTCAGCCGT	

4

Epstein-Barr virus mRNA profiles and viral DNA methylation status in nasopharyngeal brushings from nasopharyngeal carcinoma patients reflect tumor origin

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Abstract

Introduction: Undifferentiated nasopharyngeal carcinoma (NPC) is 100% associated with Epstein-Barr virus (EBV) as oncogenic driver. NPC is often diagnosed late due to initial vague complaints and obscured location. Prior studies suggest that measurement of EBV-DNA load and RNA transcripts in nasopharyngeal brushings is useful for minimally invasive NPC diagnosis. However, whether these EBV markers relate to local virus replication or reflect tumor origin remains to be demonstrated.

Methods: To resolve this, we analysed EBV-DNA characteristics and quantified latent and lytic viral RNA transcripts in nasopharyngeal brushings and matching frozen NP-biopsy specimens from patients suspected of having NPC.

Results: We observed non-fragmented and Cp-promotor methylated EBV-DNA in both nasopharyngeal brushings and biopsies suggestive of tumor origin. Using quantitative RT-PCR we determined expression levels of 7 critical latent (EBER1, Qp-EBNA1, EBNA2, BART, LMP1, LMP2, BARF1) and 5 lytic (Zta, Rta, TK, PK and VCAp18) RNA transcripts. Although latent and early lytic RNA transcripts were frequently detected in conjunction with high EBV viral load, in both brushings and biopsies the latent transcripts prevailed and reflected a typical NPC-associated latency-II transcription profile without EBNA2. Late lytic RNA transcripts were rare and detected at low levels mainly in nasopharyngeal brushings, suggestive of abortive viral reactivation rather than complete virus replication. EBV-IgA serology (EBNA1, VCAp18, Zta) did not correlate to the level of viral reactivation in situ.

Conclusions: Overall, viral RNA profiling, DNA fragmentation and methylation analysis in nasopharyngeal brushings and parallel biopsies indicate that nasopharyngeal brush sampling provides a true and robust indicator of NPC tumor presence.

What's new

NPC often is diagnosed at late stages, in part because physical examination of the nasopharyngeal space is difficult. Moreover, despite a strong association with EBV infection, optimal EBV-based diagnostic modalities for NPC are lacking. Here, EBV-DNA load, methylation status and EBV latent and lytic RNA profiles obtained from nasopharyngeal brush biopsy were found to be robustly indicative of tumor presence at the site of initiation. EBV (immediate) early mRNAs were frequently detected, suggesting local abortive viral reactivation. Using the described EBV markers, nasopharyngeal brushing may be a practical minimally invasive approach for NPC diagnosis.

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial cancer prevailing as endemic disease in southern China, Southeast Asia, the Arctic and the Mediterranean rim, including Middle East and Northern Africa[1,2]. Most endemic NPC cases classify as undifferentiated nonkeratinizing WHO type III tumors and Epstein-Barr virus (EBV) is implicated as the causal agent in the vast majority of cases[3]. EBV infects most humans worldwide leading to a longterm carrier status characterized by latent viral persistence in memory B cells in the circulation and virus shedding in saliva[4-6]. Besides in circulating B cells, EBV is maintained in a small number of epithelial cells associating with lymphoid tissue of the Waldeyer's ring, in particular the tonsils and the posterior epithelium of the Rosenmuller's fossa[7,8]. Occasionally, most likely linked to host cell differentiation, EBV may reactivate into the lytic cycle to produce virus progeny, although this process may be interrupted leading to so-called abortive replication[9-14].

During latency the viral episome is heavily methylated, limiting the number of viral transcripts being produced, whereas replicating genomes are unmethylated[3,15]. NPC tumor cells carry methylated clonal viral genomes and express the viral latency II transcription program comprising of abundant noncoding small RNAs EBER1 and EBER2, mRNAs encoding EBNA1, LMP1, LMP2A, LMP2B and BARF1 proteins and so-called BART RNAs yielding >40 microRNAs[16-18].

During virus reactivation, two viral immediate early genes (BZLF1 and BRLF1) activate the lytic cascade resulting in expression of more than 50 non-structural (early) and structural (late) proteins[3,11-14]. In vivo EBV persistence and replication is barely detectable in oral and nasopharyngeal epithelia and glandular tissues of healthy EBV carriers, except in rare conditions such as Oral Hairy Leukoplakia[19-23]. Yet, EBV is continuously shed into saliva,

even in the absence of detectable B cells, implicating non-glandular mucosal epithelia of the oro-nasopharyngeal cavity as site of virus production[5,6,19,20]. NPC is commonly diagnosed at late stages due to non-specific symptoms at early stage and a deep tumor location in the nasopharyngeal space, making NPC diagnosis by physical examination problematic[2,3]. A painful transnasal biopsy and subsequent pathological examination is commonly needed for diagnosis. Cell free EBV-DNA detected in peripheral blood of many but not all patients with NPCs, is thought to relate to DNA fragments released by apoptotic tumor cells[24,25]. However, circulating viral mRNA is virtually absent, excluding circulating tumor cells as the source of EBV-DNA[25].

Patients with NPCs show elevated IgA and IgG antibody responses to multiple viral antigens, with IgA reactivity reflecting viral activity in the oro-nasopharyngeal mucosal compartment[26-28]. Aberrant IgA antibodies against viral lytic antigens (EA and VCAp18) are detectable at early stages of NPC and in populations at risk of developing NPC[29-32]. Late and lytic antigens are essential for viral DNA replication, which is central to productive reactivation. An association between elevated IgA antibody titers, plasma EBV-DNA levels and NPC risk is strong indicating that a reactivation of EBV infection plays a role in tumor development, therefore a combination of those EBV markers is suggested for NPC screening in high-risk populations[33,34].

Molecular analysis of minimally invasive nasopharyngeal brushings that contain abnormal level of viral DNA and RNA provides an alternative and promising new approach for (early) NPC diagnosis[35-40]. The presence of latently EBV-infected NPC tumor cells in nasopharyngeal brush specimens can be visualised by cytology[41]. Furthermore, nasopharyngeal brush sampling allows assessment of tumor suppressor gene methylation, providing additional tumor markers[42-43]. In addition, following therapy, prevailing high levels of viral DNA in nasopharyngeal brushings is linked to poor response or recurrent disease[44].

Despite the potential usefulness of EBV viral load values in nasopharyngeal brushings for NPC detection, the original source of high levels of EBV-DNA concentration in nasopharyngeal brushings from patients with NPCs is not properly defined. In order to corroborate the minimally invasive nasopharyngeal brush method for robust use in NPC diagnosis, further evidence is needed to demonstrate that EBV-markers in nasopharyngeal brushings directly reflect tumor origin.

Here, we explored and confirmed the utility of nasopharyngeal brushing as an accurate and objective diagnostic tool for NPC detection by measuring EBV-DNA load, EBV latent and lytic mRNA transcripts in a multi-primed real time RT-PCR approach and by viral

DNA methylation analysis in nasopharyngeal brushings and parallel obtained frozen biopsies from the same location. The viral transcriptional activity and amount of EBV-DNA were critically analysed and expressed by means of virus content and transcriptional activity on a "per infected cell" basis. Our data suggest that nasopharyngeal brushing is a representative noninvasive tool that truly represents NPC tumor activity.

Material and methods

Patients and biological samples

Samples were collected prospectively from patients presenting with signs and symptoms suspected of NPC at the Dr. Cipto Mangunkusumo Hospital, Universitas Indonesia (Jakarta, Indonesia). For this study, the biopsy specimen was dissected into two pieces; one half was immediately snap-frozen in liquid nitrogen and the second half was fixed in buffered formalin for routine paraffin embedding and pathological examination. Biopsy, nasopharyngeal brushing, whole blood and serum sampling were carried out as described before[2,30,36].

All specimens were stored in a –80°C freezer until shipment in dry ice to the department of Pathology, VU University Medical Center (Amsterdam, The Netherlands) for DNA, RNA and serological analysis. Of the 89 nasopharyngeal brushings overall, 76 had biopsy-proven EBV-related NPC, 8 were from healthy EBV carriers and 5 were proven to have other non-EBV related head and neck diseases. From the 38 frozen biopsies used in mRNA analysis, 35 were NPC samples, 2 were taken from patient with tonsillitis and 1 was a biopsy from patient with chronic enlarged lymph node (CELN).

EBV-DNA and RNA extraction

DNA and RNA from nasopharyngeal brushings were extracted simultaneously using silica-based extraction procedure exactly as described before[45,46]. One milliliter of lysis buffer lysate was used as input for DNA/RNA isolation, and nucleic acids were eluted in 100 µl water. Reagents for silica DNA/RNA extraction were purchased as basic kit ingredients (BioMérieux). DNA and RNA isolation from frozen tissue dissolved in Trizol (5 times 10 µm sections as input) was performed according to the manufacturer's instructions (Invitrogen). DNA extracted from all materials was stored at –20°C and RNA extracts were kept at –80°C until use.

EBV-DNA load quantification by quantitative real-time PCR

The EBV-DNA load in all nasopharyngeal brushings and parallel biopsies was determined as previously described[25,37]. A 213 bp Light Cycler-based PCR assay targeting an

overlapping region of EBNA1 was performed to distinguish fragmented and intact DNA in paired brush-biopsy samples[25,36,45]. The sequences of primers and probes used for EBV markers are listed in Table 1. The cutoff value was defined at 2,321 viral DNA copies per brush as described.[36,37] EBV-DNA low and negative results in either nasopharyngeal brushings or biopsies were confirmed by spiking samples with 1000 copies EBNA1 plasmid DNA to check for PCR inhibition. During LC-PCR amplification, EBV-DNA from EBV-positive cell lines (C666.1 or Namalwa cells) was used as internal control. The EBV-DNA load in nasopharyngeal brushings was expressed as copies of EBV genome per brush; whereas in frozen biopsy samples, it was expressed as copies of EBV genome per mL Trizol lysate. Viral load in each sample was normalized to cellular β-globin DNA quantity to estimate the relative amount of EBV-DNA per cell. For each paired specimen the viral load was also expressed as number of copies of EBV genome per cell.

cDNA synthesis and quantitative RT-PCR (qRT-PCR)

Total RNA from 89 brush and 38 biopsy specimens were treated with DNase I (RQ1 RNase-free DNase, Promega) and precipitated before being used as a template to synthesize cDNA. All necessary reagents for gene specific cDNA synthesis prior to quantitative PCR including the protocol using a multi-primed approach has been described previously.[46] Of overall samples, 23 nasopharyngeal brushes and 2 biopsies, were excluded for further DNA and RNA analysis respectively, due to insufficient RNA quality, high genomic EBV-DNA contamination in cDNA (insufficient RNA purity) or low EBV-DNA per cell (limited number of tumor cells), leading to low EBER and/or negative BART and other latent transcripts.

Sixty-three out of 76 nasopharyngeal brushes from patients with NPCs, 3 out of 5 nasopharyngeal brushes from other head and neck malignancies, 33 out of 35 parallel frozen NPC biopsies, and 3 non-NPC frozen biopsies were tested for complete EBV expression profiling using a quantitative multiprimed real time LC480 Reverse Transcription Polymerase Chain Reaction (RT-PCR). Absolute quantification of EBV RNA profiles of 8 EBV latent (EBER1, BART, E1QK, EBNA2, LMP1, LMP2A, BARF1) and 5 lytic genes, consisting of the immediate early, early and late genes Zebra, Rta, PK,TK, VCAp18, was performed with a plasmid calibration for each single target as described elsewhere (Table 1) [46,48]. Real time LC-PCR reagents were purchased from Roche Diagnostics.

The qRT-PCR quantified specific cDNA relative to a validated plasmid pool containing all selected EBV latent and lytic genes in serial 1:2 dilutions in the same LC-PCR run, including the use of appropriate positive and negative controls. Amplicon length (ranging from 140 to 270 bp) and reproducibility were carefully examined by agarose gel electrophoresis. A transcript with a specific melting temperature as defined by a

Table 1. Sequences of the EBV specific primers and probes

Name	Oligo	Sequence (5′-3′)	Amplicon (bp)	Melting temp
EBV-DNA				
EBNA1	QP1-F	GCCGGTGTGTTCGTATATGG	213	
	QP2-R	CAAAACCTCAGCAAATATATGAG		
	QP3-F	CCACAATGTCGTCTTACACC	99	
	QP4-R	ATAACAGACAATGGACTCCCT		
	Donor-probe	TCTCCCCTTTGGAATGGCCCCTG		
	Acceptor-probe	ACCCGGCCCACAACCTG		
EBV-mRNA				
EBER1	EBER1-F	GAGGTTTTGCTAGGGAGGAGA	141	86.6
	EBER1-R	CGGACCACCAGCTGGTA		
BARTs	BART-F	AGAGACCAGGCTGCTAAACA	237	88.4
	BART-R	AACCAGCTTTCCTTTCCGAG		
EBNA1-QK	EBNA1-F	GTGCGCTACCGGATGGCG	236	87.0
	EBNA1-R	CATTTCCAGGTCCTGTACCT		
LMP1	LMP1-F	TGAGTGACTGGACTGGAGGA	236	82.4
	LMP1-R	GATACCTAAGACAAGTAAGCA		
LMP2A	LMP2A-F	AGGTACTCTTGGTGCAGCCC	196	83.4
	LMP2A-R	AGCATATAGGAACAGTCGTGCC		
BARF1	BARF1-F	GCCTCTAACGCTGTCTGTCC	183	87.4
	BARF1-R	GAGAGGCTCCCATCCTTTTC		
EBNA2	EBNA2-F	TACGCATTAGAGACCACTTTGAGCC	196	84.7
	EBNA2-R	AAGCGCGGGTGCTTAGAAGG		
Zebra	Zebra-F	CGCACACGGAAACCACAACAGC	143	83.2
	Zebra-R	GAAGCGACCTCACGGTAGTG		
RTA	RTA-F	TGATGATTCCTGCCACCATA	218	86.6
	RTA-R	GAGGACGGGATAGGTGAACA		
TK	TK-F	AGCTGAAgCGGCTAAGTGGGA	150	84.4
	TK-R	AGGCAGGTTTGATTACTGGTACA		
PK	PK-F	GCTGACTCCACCCACAAAAT	155	84.8
	PK-R	GAGGTCAGGCCCATGTCTAA		
VCAp18	VCAp18-F	CCAACGCGCCATAGACAAGAG	203	91.5
	VCAp18-R	GAGGCTGCTAATAGATGAAGAAACA		
Housekeeping genes				
Human b-globin	b-globin-F	GGAGAAGTCTGCCGTTACTGC	197	
	b-globin-R	TTGGTCTCCTTAAACCTGTCTTGT		
	Donor-probe	CCAGGGCCTCACCACCAACTTC		
	Acceptor-probe	CCACGTTCACCTTGCCCCACAG		
U1A	U1A-F	CAGTATGCCAAGACCGACTCAGA	226	91.1
	U1A-R	GGCCCGGCATGTGGTGCATAA		
Methylation-specific PCR (MSP)				
Methylated C-	M-F	TAACGTTTTATTTGGGAGGAGC	174	
promoter	M-R	AACAAAACGTAATTAATCCCGC		
Unmethylated C-	U-F	GAATAATGTTTTATTTGGGAGGAGT	176	
promoter	U-R	AAACAAAACATAATTAATCCCACCC		

standardized plasmid and its level above the detection limit in quantitative RT-PCR assay (800 copies per nasopharyngeal brush and 320 copies per mL Trizol NPC biopsy extract) was determined as positive. Furthermore, a transcript with a specific melting temperature yet having a very low expression level was identified as positive but unquantifiable.

As control for EBV RNA profiling each PCR amplification included cDNA prepared from RNA extracts of EBV-positive cell lines (C666.1 or Namalwa) and RNA-free water as a negative control. Control for integrity and stability of the stored RNA was performed by quantifying the absolute amount of human U1A housekeeping gene in each sample (values $>10^7$ copies per brush or biopsy were considered valid). The level of U1A transcripts was also used for RNA level normalization in addition to EBV BART mRNA which was defined as EBV-specific housekeeping transcript. Negligible signal contribution from experimental artifacts such as primer dimers and left-over genomic DNA in remaining RNA materials after DNAse treatment (defined by EBNA1 LC-PCR) were critically corrected in order to assure accuracy for viral gene expression analysis.

The amount of each EBV transcript per sample was expressed as amount of copies per brush in nasopharyngeal brushings or amount of copies per mL Trizol (per biopsy) in biopsies. The level of each transcript should be >2-fold higher than the amount of cDNA genome. Normalization was also done against the viral DNA load to estimate the amount of each transcript per EBV genome.

EBV serology

Paired serum samples available for 63 patients with NPCs were analysed for IgA antibodies against EBV-specific immunodominant epitopes of EBNA1, VCAp18 and Zebra using synthetic peptide-based ELISA assays as detailed by us previously[25,49,50]. The cutoff value (COV) was defined as mean optical density (OD450) value of 20 healthy regional controls + 2x standard deviation[49,50]. The IgA-ELISA COV for EBNA1, Zebra and VCAp18 was 0,17, 0,28 and 0.35 OD_{450} units, respectively. All sera above COV were considered positive.

Bisulfide modification and qualitative Methylation-Specific PCR (MSP)

The methylation status of the C-promoter (Cp) EBV-DNA in paired brush-biopsyspecimens was investigated by qualitative methylation-specific PCR (MSP) analysis asdescribed previously[32,51]. Bisulfite-modified DNA was amplified by two primer pairs specific for unmethylated (U) and methylated (M) Cp sequences at nucleotide positions 11041-11217 according to EBV B95.8 genome (GenBank: V01555.2; Table 1). EBV-negative BJAB cell line with 10 ng virion DNA and used as a positive control and RNA-free water as a

negative control, respectively. Ten microliters of MSP product were analyzed on a 2% agarose gel.

Statistical Analysis

Statistical analysis was executed by the SPSS version 16.0 and GraphPad Prism 6.0. EBV-DNA values between the patient and control groups and the positive rates of individual EBV gene transcripts were compared by using the Mann-Whitney test and Pearson correlation test. Linear regression was used to correlate the number of lytic transcripts with (a) the amount of EBV genome in NPC tumor biopsy and (b) level of EBV-IgA antibody response. *P* value below 0.05 was considered to be significant.

Results

We aimed to characterize EBV-DNA and RNA markers in frozen NPC tumor tissue and matching nasopharyngeal brushings, that presumably contain material of the tumor surface. Paired nasopharyngeal brush-biopsy specimens were collected prospectively from 33 patients suspected of having NPC, that were subsequently confirmed by pathological examination[37]. Clinicopathological features and molecular analysis confirmed NPC presence which allowed us to perform a complete EBV-DNA and RNA analysis. The clinical characteristics of 33 patients with NPCs with EBV markers in paired biopsy-brush specimens are summarized in Table 2.

Figure 1A shows that nasopharyngeal brushings obtained from confirmed patients with NPCs at primary diagnosis (n = 76) are 100% positive for EBV-DNA at levels above the COV compared to non-NPC controls (n = 13) (median NPC; 1.0×10^7; range from, 4.0×10^3 to 4.2×10^8 vs. median controls; 1.3×10^3; range from 0×10^3 to 6.3×10^3; Mann-Whitney test, $P < 0.0001$). We detected similarly high EBV-DNA loads in corresponding fresh-frozen NPC biopsy sections (n = 33; median, 5.9×10^6; range from 4.7×10^4 to 9.6×10^7) which agrees with data presented in previous studies[35-38].

Next, we quantified the EBV genomes per cell in paired samples from 33 patients with NPCs by normalizing the EBV viral load to the cellular human β-globin DNA load. The results show that the number of EBV genomes per cell in most of these paired samples is comparable between patients (less than 30 copies per cell; Wilcoxon T test, NS), with the exception of a few samples that seem to contain more than 50 copies per cell in either brushing or biopsy (Figure 1B). In order to define whether these high numbers represent intact EBV genomic DNA from tumor cells or chromosomal DNA fragmentation of tumor cells undergo apoptosis we performed a comparative EBV-DNA PCR analysis[25].

Table 2. Clinical characteristics of 33 patients with NPCs with EBV markers in paired biopsy-brush specimens

ID	Age (years)	Gender	TNM status	tumor stage	EBV-IgA ELISA		EBV-DNA load (10^5)			Methylation status	
					IgA-EBNA1	IgA-VCAp18	copies/ biopsy	copies/ brush	copies/ mL blood	Brush	biopsy
1	36	M	T3N0M0	3	4,3	5,0	32,0	6,9	0,0	M	M
2	48	M	T3N1M0	3	ND	ND	201,0	20,4	0,0	M	M
3	23	F	T2N2M0	3	2,7	1,3	837,0	257,6	0,2	M	M
4	25	M	T2N1M0	2	2,5	8,0	100,4	36,2	0,1	M	M
5	48	F	T3N0M0	3	13,8	4,3	3,5	152,0	0,0	M	M
6	36	F	T1N1M0	2	8,8	4,3	2,5	57,8	0,2	M	M
7	46	M	T1N3M0	4B	0,8	13,1	0,9	117,9	0,1	M	M
8	55	F	T3N1M0	3	6,9	7,9	495,0	22,7	0,0	M	M
9	28	M	T4N3M0	4B	20,0	0,7	209,0	324,0	0,0	M	M
10	56	M	T2N2M0	3	8,2	4,2	0,7	623,2	0,0	M	M
11	45	M	T2N0M0	2	1,1	1,3	964,0	129,1	0,0	M	M
12	44	M	T3N1M0	3	29,1	5,4	262,0	1183,2	0,1	M	M
13	49	M	T2N1M0	2	1,8	1,2	28,7	74,6	0,0	M	M
14	44	M	T2N3M0	4B	6,3	8,9	5,4	571,2	0,0	M	M
15	52	M	T4N3M0	4B	6,9	2,5	0,5	1089,6	0,0	M	M
16	35	M	T2N3M0	4B	11,8	21,6	566,0	924,8	0,9	M	M
17	33	M	T4N3M1	4C	0,8	1,5	140,0	4216,0	0,6	M	M
18	45	F	T2N2M0	3	2,9	1,6	2,0	372,8	0,1	M	M
19	63	M	T4N0M1	4C	4,2	1,3	199,0	108,3	0,0	M	M
20	47	F	T4N1M0	4A	1,2	0,9	87,6	547,2	0,0	M	M
21	61	M	T2N0M0	2	2,8	1,8	872,0	57,5	0,0	M	M
22	32	M	T4N3M1	4C	1,2	27,7	28,0	270,4	0,3	M	M
23	51	M	T1N0M0	1	2,1	1,1	3,2	34,9	0,0	M	M
24	55	M	T2N3M0	4B	1,8	7,7	22,7	3416,0	0,9	M	M
25	55	F	T4N3M0	4B	5,2	9,7	59,1	280,8	0,4	(U)+M	M
26	31	M	T2N2M0	3	1,9	8,6	89,4	105,1	0,0	M	M
27	31	M	T4N1M0	4A	3,2	11,5	127,4	1612,8	0,0	(U)+M	M
28	43	M	T4N3M1	4C	10,3	27,7	7,2	612,8	0,8	M	M
29	43	F	T4N3M0	4B	4,9	7,7	6,8	69,7	0,5	M	M
30	36	M	T3N2M0	3	ND	ND	226,6	304,0	0,1	M	M
31	50	M	T2N3M0	4B	4,5	6,9	891,0	571,2	0,0	(U)+M	M
32	32	M	T4N3M0	4B	3,4	5,4	40,9	166,4	0,1	M	M
33	45	F	T3N2M0	3	ND	ND	3,8	69,8	0,7	M	M

EBV-DNA load in nasopharyngeal brushings and corresponding frozen biopsies

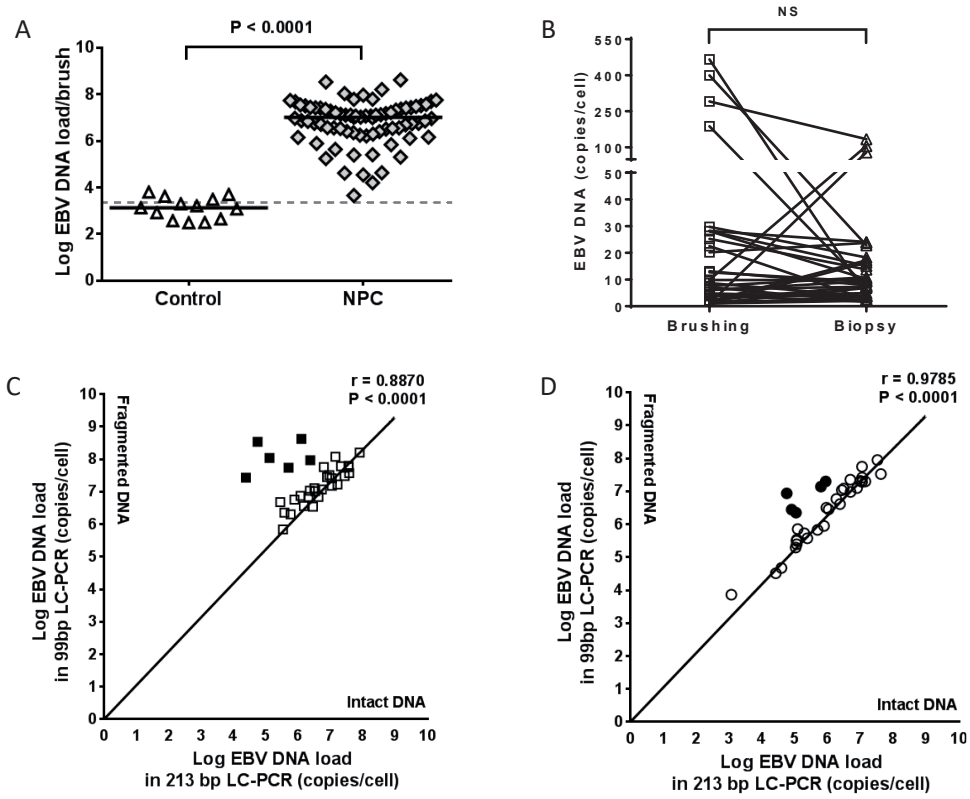

Figure 1. EBV-DNA loads in nasopharyngeal brushings and biopsies of patients with NPCs. A, A significant higher EBV-DNA load was observed in nasopharyngeal brushing obtained from patients with NPCs ($P < 0.0001$), compared to healthy and non-NPC controls. B, No significant difference between nasopharyngeal brushing and biopsy was observed in amount of EBV genome per cell detected in 33 paired NPC samples, indicating that nasopharyngeal brush sampling was a reliable method. Comparison of intact and fragmented DNA in paired brush-biopsy specimen as determined by the 213-bp and 99-bp EBV LC-PCR shows that most of DNA obtained from NPC tumor seems to remain intact despite having fragmented DNA in 6 cases. C, 27 out of 33 nasopharyngeal brushings and D, 27 out of 33 paired biopsies obtained from patients with NPCs show intact DNA, respectively (Pearson correlation; $P < 0.0001$).

The results revealed the presence of intact EBV-DNA in 27 (82%) of the 33 paired samples (Pearson correlation, r = 0.8870 for brushing and r = 0.9785 for biopsy; $P < 0.0001$). In 4 (12%) paired brush-biopsy samples, as well as in other 2 (6%) individual brushings and 2 (6%) individual biopsies, we detected higher levels of fragmented DNA that may indicate an apoptotic origin (Figure 1C, 1D). Parallel analysis of EBV-DNA load in corresponding whole blood samples of these 4 "high load" paired cases did not reveal high levels of circulating EBV-DNA genomes or DNA fragments, suggesting apoptotic extrusion rather than internal apoptotic release into the blood (data not shown).

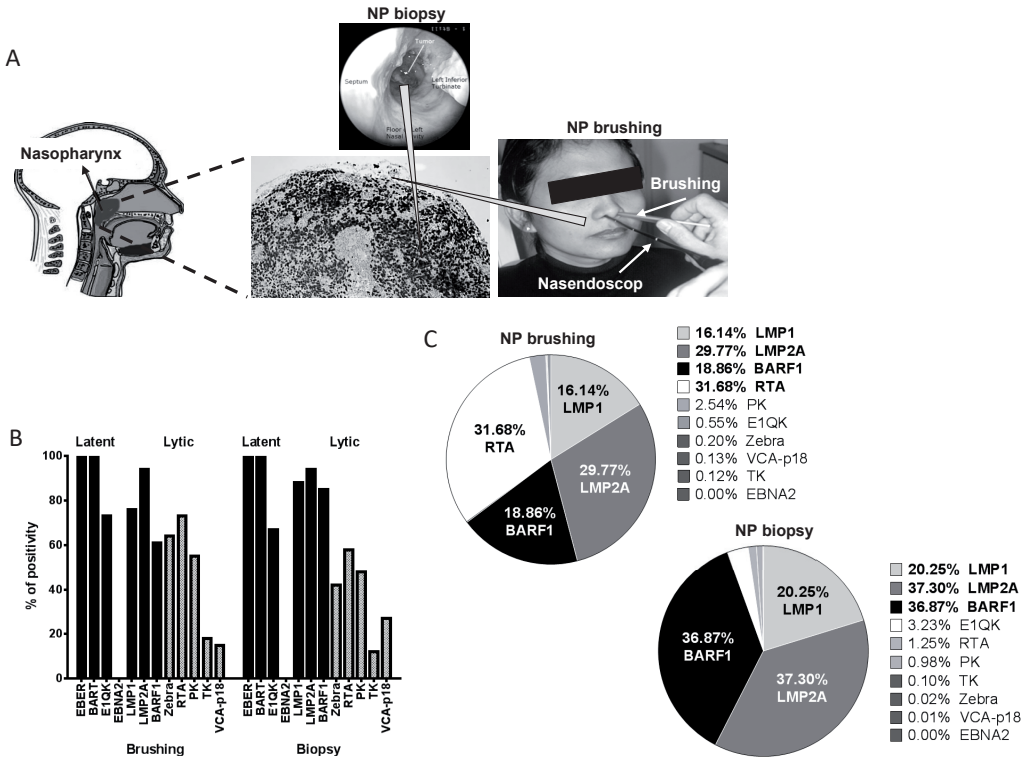

Figure 2. EBV gene expression in nasopharyngeal brushing and biopsy of NPC tumor. A, Schematic presentation of specimen collection by brush (scratching the surface of the tumor and upper mucosa) and biopsy (removal deep tissue of suspected tumor) with nasal endoscopic guidance. B, Positive detection rates of EBV latent and lytic transcripts in 33 paired brush-biopsy specimens obtained from patients with NPCs. *P* values of each EBV transcripts are depicted in Table 3. C, Percentage of EBV latent-lytic transcripts levels relative to BART RNAs in paired 33 brush-biopsy specimens. Parallel brush-biopsy samples show similar pattern of LMP1, LMP2A and BARF1 mRNA expression levels, whereas Rta mRNA is more abundant in brushings compared to biopsy samples.

EBV RNA profile in nasopharyngeal brushings corresponds to the latency II profile in biopsies

It is generally considered that NPC tumor cells express an EBV latency type II profile, although sporadic lytic transcripts have been detected as well[3,11,12,16,17]. To confirm nasopharyngeal brushing as a valid diagnostic tool for NPC, it is important to show the pattern of EBV gene expression in upper and adjoining layers of the tumor collected by nasopharyngeal brushing is similar to the pattern of EBV RNA profile in a deep location of the tumor taken by a biopsy (Figure 2). Patterns of observed viral gene expression in paired NPC brushings and biopsies are depicted in Figure 2 and Table 3.

Complete EBV latent RNA transcripts (EBER1, BART, EBNA1, LMP1, LMP2A and BARF1) were detected in 20 out of 33 (61%) paired NPC specimens (Table 3). Quantitatively, latency transcripts for LMP1, LMP2 and BARF1 prevailed in both biopsies and brusings (Figure 2B, 2C). EBNA2 transcripts could not be detected in any of NPC cases. In a subgroup of paired samples (4 to 24 out of 33 samples, depending on the transcript) additional lytic genes were detectable with immediate early and early genes (42–64% for Zebra, 58–73% for Rta and 48–55% for PK) found more frequent than other lytic markers (12–18% for TK and 15–27% for VCAp18, respectively). Samples with substantially elevated EBV genome levels (>10^6 copies per brush or per biopsy) tended to reveal qualitatively more broad viral gene expression compared to samples with low viral load (data not shown). We conclude that EBV RNA profile in nasopharyngeal brushings corresponds to the latency II profile in biopsies, with additional limited (abortive) lytic gene expression.

Positive RNA detection ratio in comparison to EBER1 and BARTs RNA presence (100%). *P* values of positive rates in paired brush-biopsy specimens were obtained by using Fisher's Exact test. *P* values for the level of positive transcript compared to the level of BART RNAs were obtained by using Mann-Whitney test. *P* value < 0.05 were considered statistically significant (*).

Tabel 3. Detection of 12 EBV-related genes in paired brush-biopsy samples

Gene	Positive cases brush n/n (%)	Positive cases of paired NPC (n = 33)		*P* value	Median transcript		*P* value
		Brushing (%)	biopsy (%)		Positive brushing	Positive biopsy	
EBER1	63/63 (100)	33/33 (100)	33/33 (100)	–	9.15e+07	2.45e+09	–
BART	63/63 (100)	33/33 (100)	33/33 (100)	–	5.26e+05	5.54e+05	–
EBNA1QK	41/63 (65)	24/33 (73)	21/33 (64)	0.847	38.60	29.14	0.312
EBNA2	0/63 (0)	0/33 (0)	0/33 (0)	–	–	–	–
LMP1	51/63 (81)	25/33 (76)	29/33 (88)	0.717	1.43	2.29	0.319
LMP2A	56/63 (89)	31/33 (94)	31/33 (94)	1.000	1.58	2.50	0.123
BARF1	48/63 (76)	20/33 (61)	28/33 (85)	0.448	5.76	1.51	0.00*
Zebra	32/63 (51)	17/33 (52)	14/33 (42)	0.670	10.39	15.01	0.675
RTA	46/63 (73)	24/33 (73)	19/33 (58)	0.564	5.21	3.11	0.788
PK	42/63 (67)	18/33 (55)	16/33 (48)	0.835	34.98	27.57	0.842
TK	14/63 (22)	6/33 (18)	4/33 (12)	0.737	74.21	136.30	0.896
VCAp18	18/63 (29)	5/33 (15)	9/33 (27)	0.388	88.15	185.50	0.789

EBV RNA quantification in nasopharyngeal brushings resembles mRNA profiles of NPC tumor biopsies

EBER1 and BART transcripts were robustly detected in both 100% of the nasopharyngeal brush and biopsy samples. As expected, EBER transcripts are extremely abundant (median EBER1 copy numbers; 9.15 x 10^7 in brushings and 2.45 x 10^9 in biopsies) and similar to the cellular U1A transcript levels (median U1A; 6.73 x 10^8 in brushings and 6.69 x 10^7 in biopsies). Moreover, the BART RNA levels were equal to the levels of the majority of latency type II (LMP1, LMP2A, BARF1) transcripts (median BART; 4.75 x 10^5 in brushings and 5.54 x 10^5 in biopsies, respectively).

When we normalized viral mRNA expression in NPC biopsies and nasopharyngeal brushings to the amount of human housekeeping U1A mRNA, we calculated extremely low levels of all viral transcripts per cell. This is best explained by a low tumor/non-tumor cell ratio in the samples (leukocyte infiltrate, stroma), typically for undifferentiated NPC. To better judge and compare the viral component between the NPC samples we also to normalized for EBV-infected (tumor) cells using BART RNA levels. BART is considered an EBV housekeeping transcript in latency II infected cells and was detected in 100% of NPC cases reflecting tumor origin (Figure 3A–3C). We also defined the level of EBV gene expression per EBV-DNA genome in each sample (Figure 3D–3F).

The transcriptional diversity in NPC EBV gene profiles in paired NPC specimens is illustrated by categorization of three groups based on expression type and level of genes; i.e. (I) latent/persistent infection, (II) sporadic lytic infection, and (III) abortive lytic reactivation. Overall, parallel brushings and biopsies showed highly similar pattern of mRNA expression levels. Abortive EBV reactivation is sporadically seen in the surface of the tumor represented by the brushing (Figure 2C). We did not detect any lytic transcripts in 5 out of 33 of total paired samples, arbitrarily defined as group I. These however expressed high levels of at least 3 latent genes (commonly expressed LMP1 and LMP2A mRNA at 10–1000 copies, whereas per BARF1 mRNA at 1–1000 copies per 100 copies BART mRNA) in both biopsy and paired brushing (representative cases shown in Figure 3A, 3D). These findings are in agreement with a previous independent study[36]. By comparison, E1QK mRNA, representing Qp driven EBNA1 transcripts, was less frequently detected in paired brush-biopsy samples and mostly present at very low expression levels (0.01–10 copies E1QK per 100 copies BART mRNA).

The second (II) group, showed expression of EBV lytic transcripts in both brushings and biopsies representing 13 out of 33 of the samples (representative cases shown in Figure 3B, 3E). In these 12 samples, 3 or more lytic transcripts were detectable in biopsies and the corresponding brushings. However, the levels of EBV lytic mRNA markers (Zebra, Rta, PK, TK, VCAp18) were generally much lower (10–1000 fold; i.e. 0.01–1 copies per 100

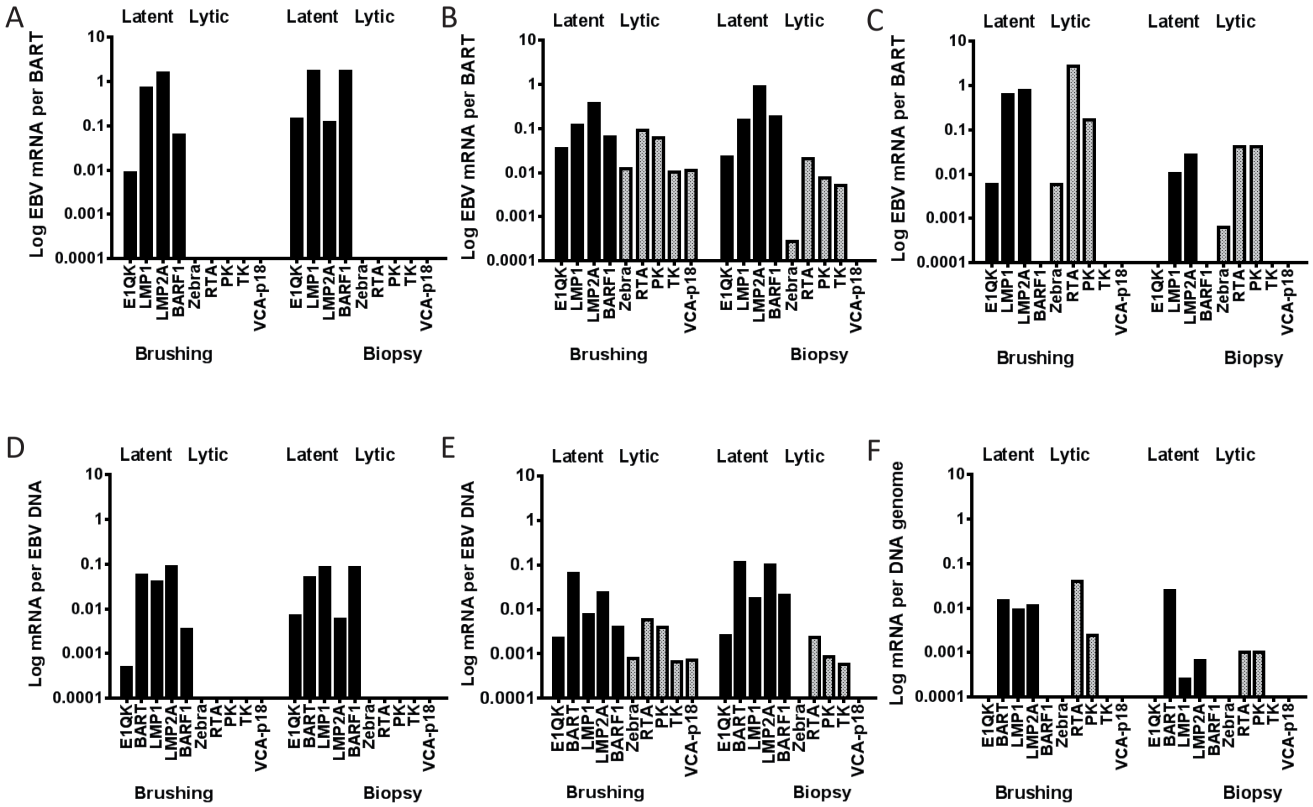

Figure 3. Expression of EBV gene profiles observed in brush-biopsy pairs of patients with NPCs relative to BART RNAs (A, B, C) and EBV genome (D, E, F) were categorized into three groups respectively. The groups consist of (A, D) truly latent/persistent infection with only latent genes (3 or more) have been expressed in high levels, (B, E) sporadic lytic infection with the expression of latent and 3 or more lytic (immediate early, early and late) transcripts were detectable in biopsies and the corresponding brushings, and (C, F) abortive lytic reactivation with both latent and lytic genes have been expressed, however only EBV immediate early and early lytic genes, but no late VCAp18 mRNA.

copies BART mRNA) than the levels of EBV latent genes (EBNA1QK, LMP1, LMP2A, BARF1) in the same sample. The late-lytic EBV transcript, VCAp18 mRNA, was barely expressed in NPC biopsies, suggesting that the full lytic EBV cycle is activated only sporadically.

In the third (III) group, 15 out of 33 samples, we detected EBV immediate early and early lytic gene expression, in the absence of late EBV lytic VCAp18 mRNA. Specifically we detected the presence Zebra and/or Rta, and PK with or without TK gene expression in both biopsy and paired brush sample (Figure 3C, 3F). Similar with the second (II) group, levels of Zebra, Rta, PK or TK varied between 0.01–100 copies per 100 BART mRNA copies. Most brushings in this group had limited Zebra expression, but revealed higher levels of Rta mRNA ranging from 10–1000 copies per 100 copies BART mRNA.

Rta mRNA is more frequently detected (12/15) and present at higher levels compared to Zebra mRNA (8/15). Because in this group PK and TK were barely expressed and VCAp18 mRNA was absent, we conclude that the reactivation patterns reflect abortive (partial) viral reactivation. This comprehensive quantitative analysis strongly suggests that both latent and lytic transcript expression in NPC biopsies is represented equally and reliably in nasopharyngeal brushings.

EBV (sporadic) reactivation is poorly related to EBV-DNA genome levels

It is postulated that physiological signals *trigger* EBV reactivation *and increase* oral shedding[5,6,9,10,12]. We wonder whether the observed sporadic EBV lytic reactivation might cause high viral loads in the mucosal layer, as collected in the brushings. To this end we compared the presence and number of detectable EBV lytic transcripts to the amount of EBV-DNA per cell in a total of 57 nasopharyngeal brushings and 27 parallel primary tumors (Figure 4). Six samples containing high levels of fragmented DNA (Figure 1C, 1D) were discarded for this analysis. Interestingly, the detection of lytic transcripts indeed positively correlated with the levels of EBV-DNA (copy number per cell) in the nasopharyngeal brushings (Figure 4A), while this was not observed for the biopsies (Figure 4B). This observation suggests that the sporadic lytic events are localized to the surface of NPC tumors which are sampled in the nasopharyngeal brushing. However, because expression of immediate early and early genes (1–4 lytic transcripts in nasopharyngeal brushings and 1–3 lytic transcripts in biopsies) prevailed over the complete late lytic genes including VCAp18 (3–5 lytic transcripts), it remains unclear what cell type or types contribute to the EBV-DNA signal.

Overall these data indicate that the superficial layer of NPC may sustain an abortive reactivation in infected tumor cells rather than a full viral replication as seen in healthy epithelial cells lining secondary lymphoid tissues in the nasopharynx such as the tonsils.

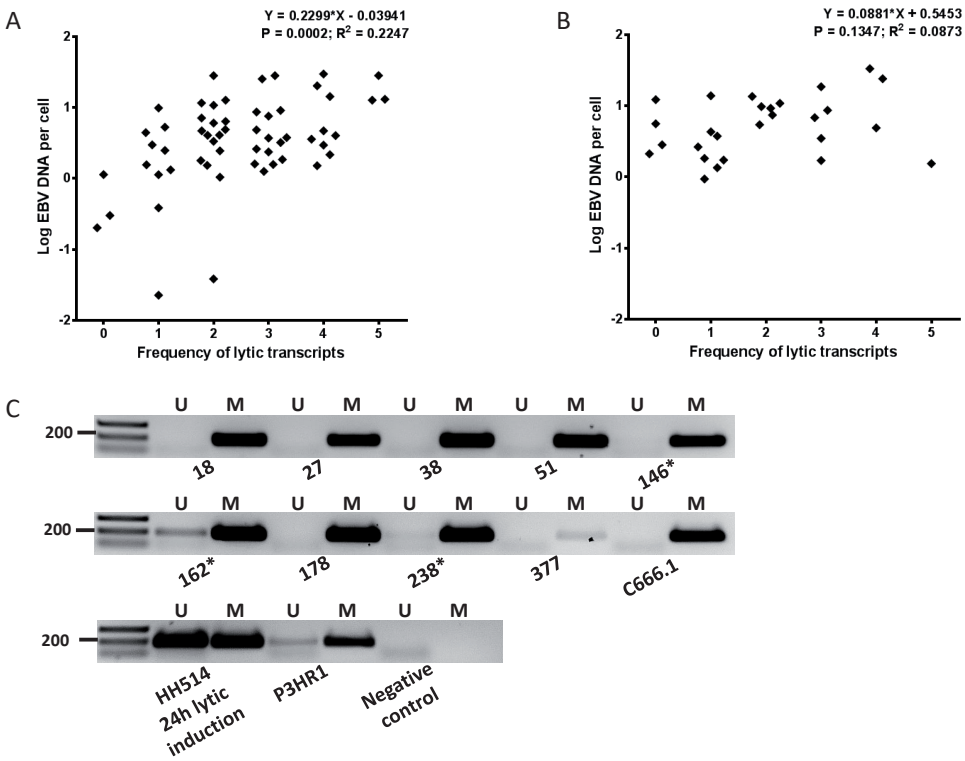

Figure 4. EBV (sporadic) reactivation in relation to EBV-DNA genome level shows A, a correlation trend in 57 nasopharyngeal brushings (Linear regression, r = 0.2299 ± 0.0576; *P* = 0.0002), but B, no significant correlation observed in parallel biopsies (Linear regression, *P* = 0.1347). C, All brush-biopsy pairs reveal methylated EBV-DNA at the latency-associated C promoter (Cp) reflecting tumor origin (Table 2), whereas partly unmethylated Cp DNA is detected in only 3 nasopharyngeal brushings indicated by (*). Heavily methylated EBV genome of C666.1 cells, mixed methylated and unmethylated EBV-DNA of lytically induced HH514 cells and partly unmethylated EBV genomes of P3HR1 cells were used as positive controls.

We conclude that the EBV genome copies measured in the nasopharyngeal brushings most likely represent extruding tumor cells with reactivating EBV captured in the mucosa that are then scraped from the tumor surface.

EBV genome in nasopharyngeal brushings is predominantly methylated

In order to distinguish non-methylated EBV virion DNA from methylated tumor-associated EBV-DNA we then analysed the methylation status of the C promoter (Cp) region at nt 11041-11217 on the EBV genome in all 33 biopsies and paired nasopharyngeal brushings[3,15,19]. We found methylated Cp DNA in 100% of the biopsy samples and only partially unmethylated Cp DNA in 3 nasopharyngeal brushings (Table

2, Figure 4C). The results are compatible with the tumor origin of EBV-DNA in the NP-brush specimens and indicate presence of only sporadic low level lytic replication in the superficial layers of some tumors, as collected with the nasopharyngeal brushing. No correlation was found between EBV-DNA load and methylation status of EBV-DNA (Table 2), eliminating viral lytic replication as source of EBV-DNA and confirming the predominant tumor cell origin of DNA in the nasopharyngeal brushings.

EBV antibody responses is not correlated to EBV (sporadic/partial) reactivation

We hypothesized that reactivation of EBV lytic cycle gene expression within the NPC tumor milieu might stimulate mucosal antibody responses against immunodominant epitopes of the tumor-released latent antigen (EBNA1) and the early plus late lytic cycle proteins (Zebra and VCAp18). Therefore we analyzed EBV antigen-specific IgA seroreactivity by synthetic peptide-based ELISA in 63 paired serum samples from patients with NPCs collected at primary intake in parallel to the NP-brush and biopsy.

All sera (100%) showed elevated IgA antibody responses above cutoff against EBNA1 and VCAp18. IgA-EBNA1 antibody reactivity is unrelated to the presence and level of EBV latent gene expression (data not shown) or lytic gene expression in nasopharyngeal brushings (Figure 5A; Kruskal-Wallis/Dunn's test, NS). IgA-EBNA1 reactivity is also unrelated to the EBV-DNA load in nasopharyngeal brushings (data not shown). Aberrant IgA antibodies to VCAp18 (Figure 5B), reflecting exposure to late lytic gene products of EBV was also unrelated to the presence and number of lytic transcripts in nasopharyngeal brushings (Kruskal-Wallis/Dunn's test, NS), despite high levels of Zebra and Rta mRNA or high EBV genomes per cell equivalent being present in in some cases.

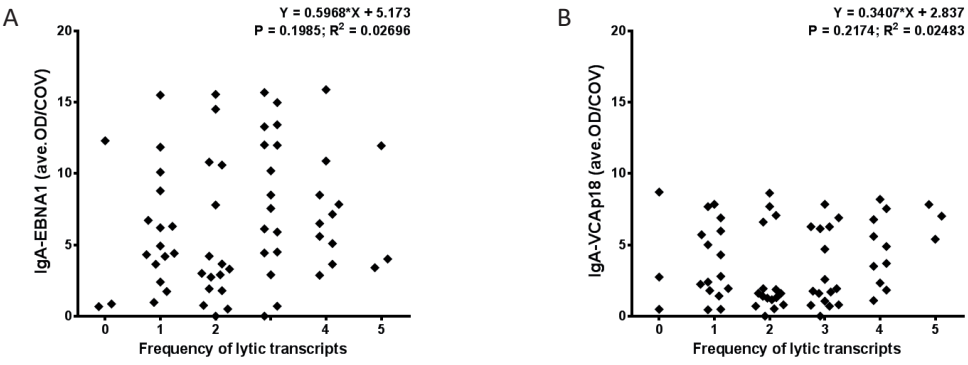

Figure 5. EBV (sporadic/partial) reactivation is unrelated to aberrant IgA antibodies either against A, EBNA1 (Linear regression, $P = 0.1985$) or B, VCAp18 (Linear regression, $P = 0.2174$), respectively.

Elevated IgA-Zebra antibody levels were also detectable in most patients, but the level of IgA-Zebra reactivity is neither related to the number of lytic transcripts in brushings nor to the expression level of immediate early genes Zebra and Rta (data not shown). Thus, overall the EBV IgA antibody responses are independent and quantitatively unrelated to local EBV reactivation as detectable in NPC brushings and biopsy tissues.

Discussion

The nasopharyngeal brushing has been proposed as an alternative for the invasive and painful biopsy in detecting the presence of NPC at the primary site of tumor development[35-40]. Nasopharyngeal brushings collected from patients with NPCs carry high levels of EBV-DNA and carcinoma-specific BARF1 mRNA reflecting the presence of viable tumor cells in the NP epithelium (Figure 2A) [17,35,36,41]. Methylation analysis of defined cellular tumor suppressor genes can confirm the presence of tumor cells in nasopharyngeal brushings[3,42,43,52]. Furthermore, changes in brush EBV-DNA levels directly reflect the tumor response to treatment and can be used to identify local recurrent disease[37,40,44].

Our data confirm and extend previous studies revealing highly elevated EBV loads in nasopharyngeal brushings from patients with NPCs as compared to controls[35-40]. We found that the number of viral genomes per cell as well as the pattern of overall EBV latent and lytic gene expression profiles are comparable in parallel brush-biopsy specimens, confirming the diagnostic value of nasopharyngeal brushings[3,36].

Prior molecular analysis of NPC tumor biopsies mainly revealed EBV gene products reflecting the latency type II transcription profile with no or limited viral lytic activity[3,11,16,17,53]. Some EBV infected tumor cells may express lytic gene products sporadically due to epithelial cell differentiation leading to either full or abortive viral reactivation, which remains to be defined[9-13]. In this study we analysed multiple EBV genes reflecting latent and lytic viral activity in nasopharyngeal brushing and biopsy specimens, sampled under nasendoscopic guidance during primary diagnostic assessment at the site of suspected NPC presence. Both NPC brush and biopsy samples revealed abundant EBV latency II gene transcripts, without EBNA2 mRNA (Figure 2, 3).

Quantitative EBV transcript profiling revealed that LMP1, LMP2A and BARF1 mRNAs are most prevalent in up to 80% of cases in both brush and biopsy samples, consistent with prior reports[3,16,17,52,53]. We recently confirmed that BARF1 also acts as early lytic transcript, activated by Rta[54]. BARF1 mRNA is prevalent in overall brush-biopsy samples irrespective of lytic gene expression, reflecting its character as latent transcript (Figure

2, 3A, 3D). Rta mRNA is expressed particularly in nasopharyngeal brushings (Figure 2C), reflecting initiation of lytic cycle in superficial tumor cells protruding into the mucosal layer, possibly enhancing BARF1 expression above the existing latency level (Figure 2, 3 and Table 3). Therefore, we conclude that BARF1 mRNA mainly is a latency-associated gene product in NPC and forms a suitable NPC marker, as suggested before[17,36].

The high and stable expression of BARTs in NPC biopsies and brushings is consistent with prior data and classifies the BART transcript as EBV housekeeping gene, suitable for specific normalization of EBV-RNA profiles[34,53,55,56]. Relative to BART RNA levels, EBV latency II transcripts prevail over immediate early and early lytic transcripts (Figure 2C). Late lytic transcripts are present sporadically and only at low level, reflecting limited and mostly abortive viral replication in the mucosal tumor surface.

Profiling of EBV lytic infection in nasopharyngeal brushings showed a more heterogeneous pattern than previously appreciated. The higher frequency of (immediate) early mRNAs, and the low level or absence of late lytic transcripts in most samples suggest that the majority of EBV reactivations may be defective (abortive reactivation). Lack of full virion production is strongly confirmed by the absence of unmethylated virion DNA in all biopsies and brushings except 3 brush samples showing partially unmethylated DNA (Table 2, Figure 4C).

The cases with extremely high copy number of EBV genomes per cell in brush and biopsy may reflect accumulation of apoptotic fragments shed from mucosal epithelial cells, involving a unique cellular mechanism called epithelial extrusion[57]. Epithelial cell migration is induced by LMP1 and dying cells may be extruded during NPC tumorigenesis. However whether a high level of fragmented EBV-DNA in nasopharyngeal brushings is related to epithelial extrusion remains unknown[58,59].

Highly abundant viral DNA content in nasopharyngeal brushings is considered to derive from tumor cells collected at the site of primary tumor formation. Alternatively, viral DNA may derive from new virions produced by latently infected tumor cells undergoing EBV replication switch at superficial mucosal epithelia. In healthy EBV carriers, the virus reactivates in the oropharynx, continuously releasing virions into the saliva, even in the absence of circulating B cells[5,6,20].

The role of oro-/nasopharyngeal epithelial cells in EBV lytic infection is controversial except in rare conditions such as Oral Hairy Leukoplakia where productive EBV replication occurs in the superficial differentiating *epithelial* layers[21-23]. The predominance of methylated EBV Cp-DNA sequences in nasopharyngeal brushings, supports the notion

that EBV-DNA in the brush originates from tumor cells expelled into the nasopharyngeal mucosal layers, rather than from locally produced virions (Table 2, Figure 4C).

Fragmented EBV-DNA derived from apoptotic tumor cells is also considered to disperse into the peripheral blood as detected in many but not all patients with NPCs[24,25]. Intact (infectious) virions and full length viral genomes are not detected in the circulation of patients with NPCs. Our data indicate that latent, abortive or full lytic EBV gene expression profile in either biopsy or nasopharyngeal brushing of the same patient are not related to the viral loads (Figure 4) nor to circulating anti EBV-IgA antibody levels (Figure 5). This suggests that EBV-IgA responses are triggered by viral replicative events outside the tumor tissue preceding and progressing in parallel with tumor formation. This is in line with the observation that elevated VCA-IgA levels are generally present prior to appearance of a detectable tumor mass.

Previously we and others showed that the molecular diversity of EBV-reactive IgG and IgA responses varies greatly among patients with NPCs, reflecting heterogeneous EBV antigen exposure during NPC development[26-30]. We suggest that VCA-IgA reactivity reflects loco-regional virus replication events outside the initiating tumor mass, potentially contributing to tumor initiation by providing infectious EBV to infect susceptible cells capable of maintaining the viral genome, as recently identified[60,61]. Recently, the combination of EBV-IgA serology with EBV-DNA load analysis in nasopharyngeal brushings has been proposed as preferred method for population screening programs to identify early stage NPC[36,38-40].

Whether active virus replication also occurs in sporadic malignant epithelial cells or in submucosal lymphoid cells remains unclear since histochemical staining failed to reveal a cellular niche of local lytic producer cells[12,19]. The exact location of epithelial cells as the natural site of viral replication in the oro-nasopharyngeal region remains unanswered. The data in this study confirm the validity of using nasopharyngeal brushing to assess aberrant EBV biomarkers reflecting NPC presence and activity at the site of tumor initiation.

Conclusions

Nasopharyngeal brushing may provide an effective and practical minimal invasive tool for NPC diagnosis, with EBV providing valid tumor markers in the form of viral DNA load or specific RNA. These findings may encourage the transfer of NP brush based diagnostics to the clinic.

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5

Quantitative multi-target RNA profiling in Epstein-Barr virus

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Abstract

Introduction: Epstein-Barr virus (EBV) is etiologically linked to multiple acute, chronic and malignant diseases. Detection of EBV-RNA transcripts in tissues or biofluids besides EBV-DNA can help in diagnosing EBV related syndromes. Sensitive EBV transcription profiling yields new insights on its pathogenic role and may be useful for monitoring virus targeted therapy.

Methods: Here we describe a multi-gene quantitative RT-PCR profiling method that simultaneously detects a broad spectrum (n = 16) of crucial latent and lytic EBV transcripts. These transcripts include (but are not restricted to), EBNA1, EBNA2, LMP1, LMP2, BARTs, EBER1, BARF1 and ZEBRA, Rta, BGLF4 (PK), BXLF1 (TK) and BFRF3 (VCAp18) all of which have been implicated in EBV-driven oncogenesis and viral replication.

Results: With this method we determine the amount of RNA copies per infected (tumor) cell in bulk populations of various origin. While we confirm the expected RNA profiles within classic EBV latency programs, this sensitive quantitative approach revealed the presence of rare cells undergoing lytic replication. Inducing lytic replication in EBV tumor cells supports apoptosis and is considered as therapeutic approach to treat EBV-driven malignancies.

Conclusions: This sensitive multi-primed quantitative RT-PCR approach can provide broader understanding of transcriptional activity in latent and lytic EBV infection and is suitable for monitoring virus-specific therapy responses in patients with EBV associated cancers.

Highlights

- Multi-primed quantitative RT-PCR is suitable for accurate quantification of RNA molecules enabling standardization.
- Viral RNA profiling revealed the expected RNA expression patterns according to the latency program and presence of reactivating EBV.
- Quantification of 16 viral targets enables monitoring of EBV behaviour in malignancies during therapy as biomarkers for therapy response.

Introduction

Epstein-Barr virus (EBV) is a human γ1-herpesvirus 4 (HHV4) with tropism for lymphoid and epithelial cells, as reflected in the nature of EBV associated autoimmune diseases and distinct malignancies, including Burkitt's and various (non-)Hodgkin's lymphomas, post-transplant lymphoproliferative disease and HIV-associated lymphomas, as well as gastric and nasopharyngeal carcinomas. Although the viral genome potentially encodes more than 80 proteins, EBV has a highly restricted viral gene expression during latency and in tumor cells[1,2].

Distinct latency programs are used by EBV for driving initial transformation of newly infected B cell (latency type III), mimicking the germinal center reaction (latency type II), and allowing "normal" viral persistence in resting memory B cells (latency type 0), the latter showing only non-coding RNA expression. During cell division of memory B cells, EBNA 1 (latency type I) is expressed in addition to the non-coding RNAs to maintain the viral genome[3]. Activation and differentiation of an EBV-carrying B cell into plasma cell triggers viral lytic cycle gene expression[3-5]. The diversity in latency programs is most clearly reflected in EBV driven malignancies and regulated by different promoters. EBV latency gene products actively contribute to viral persistence and are linked to oncogenesis by manipulating multiple cellular pathways controlling growth, differentiation, apoptosis and homeostasis[6,7]. Latency type III results in expression of six nuclear proteins (EBNAs 1-6), two membrane-associated proteins (LMP1 and LMP2), non-coding EBERs (EBER1 and 2) and BARTs transcripts, the latter being precursors for up to 40 distinct viral microRNAs[1,8]. Since the expression of the EBNA2-6 proteins induce strong immune responses, latency type III is only observed during initial phase of primary infection and in lymphomas arising in immune compromised patients.

In EBV driven (non-) Hodgkin's lymphoma, gastric and nasopharyngeal carcinomas a more restricted latency pattern II is observed in which LMP1 (absent in gastric

carcinoma), LMP2 and BARF1 (absent in lymphoma), are expressed besides EBNA1 and non-coding EBERs and BARTs. In EBV-positive lymphoma and carcinoma tissue as well as in tumour-derived cell lines and in EBV carrying circulating memory B cells, most of the viral genome is heavily methylated and the non-methylated Q promoter drives expression of EBNA1, which is crucial for viral genome maintenance in dividing cells[9].

During initial B cell infection and transformation an additional protein and 3 microRNAs are expressed that originate from the BHRF1 region. The BHRF1 open reading frame encodes a viral Bcl2 homologue[10], which is expressed as a latent protein in growth-transformed cells in vitro, but also in some Wp-restricted Burkitt's lymphoma in vivo possibly contributing to tumorigenesis by inhibiting apoptosis[11]. Otherwise the BHRF1 encoded viral Bcl-2 protein homologue is mostly known as being expressed during the viral lytic (replicative) cycle[12].

In EBV-transformed lymphoblastoid cell lines (LCL) EBNA1 is encoded from the 3'-end of a long precursor mRNA processed by differential splicing and expressed from either the Wp or the Cp promoter[13]. In both lymphoid and epithelial tumour tissue in vivo, and related cell lines cultured ex vivo, Wp/Cp are switched off by methylation and EBNA1 RNA transcription originates from the non-methylated Q promoter. In the lytic phase yet another promoter is used for transcription of EBNA1, the F promoter[14,15].

In EBV driven tumours viral transcription is largely limited to defined latency type[7]. However sporadic lytic cells may be present, which[16,17] according to some studies may contribute to lymphomagenesis[18]. The transition from latency to the reproductive phase of EBV is tightly regulated by the "immediate early" transcription factors ZEBRA (Zta; BZLF1) and R (Rta; BRLF1), which are essential for inducing the lytic cascade associating with expression of the viral DNA replication machinery[19]. Newly formed non-methylated viral genomes are a template for production of late mRNAs encoding structural proteins, like viral capsid proteins (eg. VCAp18, BFRF3) and membrane glycoproteins (gp125, gp350/220; BALF4 and BLLF1, respectively), enabling the assembly and spread of new infectious virions[20].

The viral "early" antigens include enzymes essential for nucleotide metabolism and DNA replication, involving two viral kinases, Protein Kinase (PK, BGLF4) and Thymidine Kinase (TK; BXLF1), which are used as therapeutic targets since they can convert antiviral prodrugs (eg. (val)ganciclovir) to the active tri-phosphorylated form, which subsequently blocks viral DNA replication and inhibits late mRNA synthesis[21-23]. Quantitative analysis of lytic transcripts was used in previous studies to prove the mechanism of tumor treatment by viral lytic induction and subsequent tumor cell killing by antiviral drugs (ganciclovir)[24,25].
EBV transcriptome activity has been widely studied in EBV infected lymphoid and

epithelial cells lines to elucidate the role of EBV in tumorgenesis, as they reflect the expression profiles in patient material derived from blood leukocytes or tissue sections of different EBV related malignancies[26-33]. However a broader use of transcriptomics was hampered by difficulties to compare studies due to a lack of standardization relative to different cell lines[11,26,28].

A first attempt for standardization of EBV profiling was performed by using in vitro transcribed RNA as standard for each target gene[34]. To circumvent this, more recent studies use micro array approaches[33] and RNA-seq or Nanostring based techniques which result in well validated transcription profiles[31,32]. These methods need more complex laboratory and bioinformatic skills, whereas for diagnostic use a standardized method with an easy to interpret readout would be prefered. The need for accurate validation of (therapy-induced) changes in gene transcription would require an absolute quantification method. Recently a method for absolute quantification was developed using a 48:48 dynamic array IFC assay. Quantification was performed by a single plasmid containing a single copy of all target genes[35].

We here describe a non-biased quantitative multi-primed RT-PCR to determine the number of RNA molecules of 16 viral and one cellular gene. Absolute quantification was achieved during PCR by using a standard curve derived from a plasmid pool containing all targets. This highly accurate and standardized method enables a detailed and robust analysis of relevant RNA profiles of all EBV associated malignancies.

A cross examination of commonly used EBV positive cell lines of lymphoid and epithelial origin revealed that our generated RNA profiles largely matched the known latency types. However, we found that the sensitivity of the quantitative RT-PCR here described allowed the detection of low level of lytic cells as well, confirming recent findings[31,32,35]. This method proved useful in monitoring virus-specific lytic-induction therapy responses in EBV associated malignancies[24,25]. Accurate RNA quantification extends our understanding of the role of EBV infection in different diseases and will be useful in monitor EBV activity in clinical samples at diagnosis and following treatment.

Material and methods

Cell culture

EBV negative cell lines included Burkitt lymphoma cell lines; BJAB, Mutu9, AK31 (provided by M. Ressing), the nasopharyngeal carcinoma cell line HONE-1 and the gastric carcinoma cell line AGS. EBV positive cell lines were Burkitt's lymphoma latency

type I (Akata, Daudi, P3HR1 and HH514), Burkitt's lymphoma latency type III (Raji, Jijoye and Namalwa). Well studied lymphoblastoid cell lines (LCLs) established with the B95.8 type-I EBV strain (X50-7, IB4, JY and RN) or the AG876 type-II strain (JC5) were used as well. IM1 is a spontaneous LCL line obtained from an individual with Infectious Mononucleosis.

The EBV nasopharyngeal carcinoma C666.1 was kindly provided by D. Thorley-Lawson. AGS-BX1 is a gastric cell line containing the Akata recombinant strain of EBV (kindly provided by L. Hutt-Fletcher) and SNU719, a naturally derived EBV-infected gastric carcinoma cell line (purchased from Korean Cell Bank). Three spontaneous wt-LCL (IK, RI and MU) were generated by in vitro outgrowth of peripheral blood mononuclear cells from healthy individuals in the presence of 1 mM Cyclosporine-A (kindly provided by R. Khanna). We generated low passage BLCL2 and 3 by freshly culturing B cells from 3 different Caucasian donors in the presence of EBV B95.8. All lymphoid cells were cultured in RPMI-1640 medium, supplemented with 10% FCS and 100 µg/mL penicillin, 100 IU/mL streptomycin and 1mM glutamine (P/S/G).

For the induction of the lytic cycle in HH514, cells were treated with 20 ng/mL TPA and 3 mM butyric acid at 32°C for 24 h[36]. AGS-BX1 cells were cultured in Ham's F12 nutrient mixture (Lonza) supplemented with 10% FCS and P/S/G. BX1-EBV virus was maintained in the AGS background by selection with 400 µg/mL geneticin (Gibco). C666.1 were cultured in DMEM with 10% FCS and P/S/G using bovine fibronectin (Calbiochem) coated flasks. SNU719 cells were cultured in RPMI with 10% FCS and P/S/G. EBV lytic cycle gene expression in the carcinoma cells was induced by 3 µM gemcitabine (GCb) and 0.3 mM valproic acid (VPA), as described elsewhere[24].

Plasmids

Plasmids representing each target gene were created and used as standard to quantify RNA levels. Vectors containing BamHI fragments were kindly provided by T. Ooka for the targets, BARF1 (BamHI A), TK (BamHI X), and VCAp18 (BamHI F). The ZEBRA and RTA vector (pSP64 and sg5-R) were kindly provided by P Farrell and SD Hayward, respectively. EBNA2 and BARF0 encoding the BART constructs were previously described by Brink et al[37]. For generation of plasmids containing the targets, RNA was isolated from JY cells. cDNA was synthesized for EBER1, EBNA1 Y3K, EBNA1 QK, EBNA1 coding domain sequence (CDS), EBNA2, LMP2, BHRF1 Y2H3 and PK, using the reverse primers of each target (Table 1).

For the lytic transcript BHRF1 H2H3, RNA was isolated from B98.5 cells induced with TPA. After PCR amplification with the primers described in Table 1 using Phusion DNA

polymerase (Finnzymes), amplicons were cloned into the pCR-Blunt II-TOPO vector (Invitrogen). The construct for human Small Nuclear Ribonucleoprotein Polypeptide A SNRPA (also called U1A) was derived similarly from Hela S3 cells, but cloned into the PCR II vector (Invitrogen)[38]. The template for PCR for LMP1 was the vector pcDNA3.1-LMP1 (provided by J. Tellam). Insertions were confirmed by DNA sequence analysis. Upon purification of plasmids by the plasmid mini prep kit (Qiagen), the number of DNA molecules was calculated based on DNA concentration measured by Nanodrop and the molecular weight (nucleotide content) of the specific plasmids. A plasmid pool was prepared as a mix of 10^8 copies per µL of each target and stored in small aliquots at –80°C. This stock was used to make a working stock by 100-fold dilution and further serial 10-fold dilutions were used in the PCR to obtain a standard curve for quantification.

Synthesis of RNA run-off transcripts

All RNA experiments were conducted under strict RNAse-free conditions. Plasmids containing specific inserts for each mRNA target as described above were cut at the end of the target gene with the appropriate restriction enzyme to linearize the plasmid providing a stop for transcription. DNA was purified and used for generating sense strand RNA run-off transcripts (ROT) by SP6 or T7 RNA polymerase (Promega). The ROT were DNase1 treated by RQ1 RNAse free DNAse1 (Promega), purified by RNeasy Mini Kit according to manufacturer's protocol (QIAgen) and analyzed by agarose gel electrophoresis. The RNA concentration was determined by Nanodrop and the amount of RNA molecules was calculated by the molecular weight of the nucleotide content of the ROT. Purified RNA stocks of 10^8 and 10^{10} copies/µL were stored in appropriate aliquots at –80°C until use.

RNA isolation from cultured cells

Total RNA was extracted from 250,000 cells with >95% viability (Trypan Blue exclusion) harvested in the exponential phase of growth using 0.25 mL TRIzol reagent (Invitrogen Life Science). Subsequently RNA was treated with RQ1 RNase free DNase1 (Promega) according to manufacturer's protocol. RNA was precipitated at –80°C with 0.1 volume 3M NaAc pH 5.3, 2.5 volumes EtOH and 2.5 µg linear acryl amide (Ambion), centrifuged, and washed with 70% ethanol and the pellet was air dried, eluted in 20 µL H2O and stored as above.

Real time quantitative RT-PCR

A quantitative RT-PCR was created for the detection of EBNA1 (Y3K, QK splice variants, and in the coding sequence (CDS), EBNA2, LMP1, LMP2, EBER1, BARTs, BHRF1 (latent and lytic variant), BARF1, ZEBRA, RTA, TK, PK and VCAp18 RNA. cDNA synthesis was performed on purified RNA by addition of 5 µl multi-primer mix (consisting of 14 reverse

Table 1. Overview of primer sequences

EBV-target	Name	Genomic coordinates (AJ507799)	Sequentie (5′-3′)	Amplicon Length (bp)	Melting temp (°C)
EBER1	EBER1-F	6647-6667	GAGGTTTTGCTAGGGAGGAGA	141	86.6
	EBER1-R	6787-6766	CGGACCACCAGCTGGTA		
BARTs	BART-F	156696-156715	AGAGACCAGGCTGCTAAACA	237	88.4
	BART-R	158736-158717	AACCAGCTTTCCTTTCCGAG		
EBNA1	EBNA1-Y3F	36109-36128	TGGCGTGTGACGTGGTGTAA	265	86.6
	EBNA1-QF	50153-50169	GTGCGCTACCGGATGGCG	236	87.0
	EBNA1-R	95698-95679	CATTTCCAGGTCCTGTACCT		
	EBNA1-CDSF	96888-96907	AGGGAAGCCGATTATTTG	191	88.9
	EBNA1-CDSR	97078-97059	CTCCTTGACCACGATGCTTT		
LMP1	LMP1-F	168886-168867	TGAGTGACTGGACTGGAGGA	236	82.4
	LMP1-R	168497-168507 /168584-168593	GATACCTAAGACAAGTAAGCA		
LMP2	LMP2-F	751-770	AGGTACTCTTGGTGCAGCCC	196	83.4
	LMP2-R	1102-1081	AGCATATAGGAACAGTCGTGCC		
EBNA2	EBNA2-F	35609-35633	TACGCATTAGAGACCACTTTGAGCC	196	84.7
	EBNA2-R	36190-36171	AAGCGCGGGTGCTTAGAAGG		
BHRF1 latent	BHRF1-Y2F	35609-35633	TACGCATTAGAGACCACTTTGAGCC	249	83.1
	BHRF1-R	42192-42173	TTCTCTTGCTGCTAGCTCCA		
ZEBRA	ZEBRA-F	90396-90375	CGCACACGGAAACCACAACAGC	143	83.2
	ZEBRA-FB*	90396-90375	ACGACGCACAAGGAAACC	147	83.0
	ZEBRA-R	90046-90053/ 90138-90149	GAAGCGACCTCACGGTAGTG		
Rta	RTA-F	92507-92488	TGATGATTCCTGCCACCATA	218	86.6
	RTA-R	92290-92309	GAGGACGGGATAGGTGAACA		
BARF1	BARF1-F	165414-165433	GCCTCTAACGCTGTCTGTCC	183	87.4
	BARF1-R	165596-165577	GAGAGGCTCCCATCCTTTTC		
BHRF1 lytic	BHRF1-H2F	41542-41561	GTCAAGGTTTCGTCTGTGTG	211	83.7
	BHRF1-R	42192-42173	TTCTCTTGCTGCTAGCTCCA		
TK	TK-F	131869-131849	AGCTGAAGCGGCTAAGTGGGA	150	84.4
	TK-R	131720-131742	AGGCAGGTTTGATTACTGGTACA		
PK	PK-F	110390-110371	GCTGACTCCACCCACAAAAT	155	84.8
	PK-R	110236-110255	GAGGTCAGGCCCATGTCTAA		
VCAp18	VCAp18-F	49429-49448	CCAACGCGCCATAGACAAGAG	203	91.5
	VCAp18-R	49629-49605	GAGGCTGCTAATAGATGAAGAAACA		
U1A	U1A-F	-	CAGTATGCCAAGACCGACTCAGA	226	91.1
	U1A-R	-	GGCCCGGCATGTGGTGCATAA		

*A different ZEBRA primer is used for carcinomas (Z1B) due to a single mutation (shown in bold).

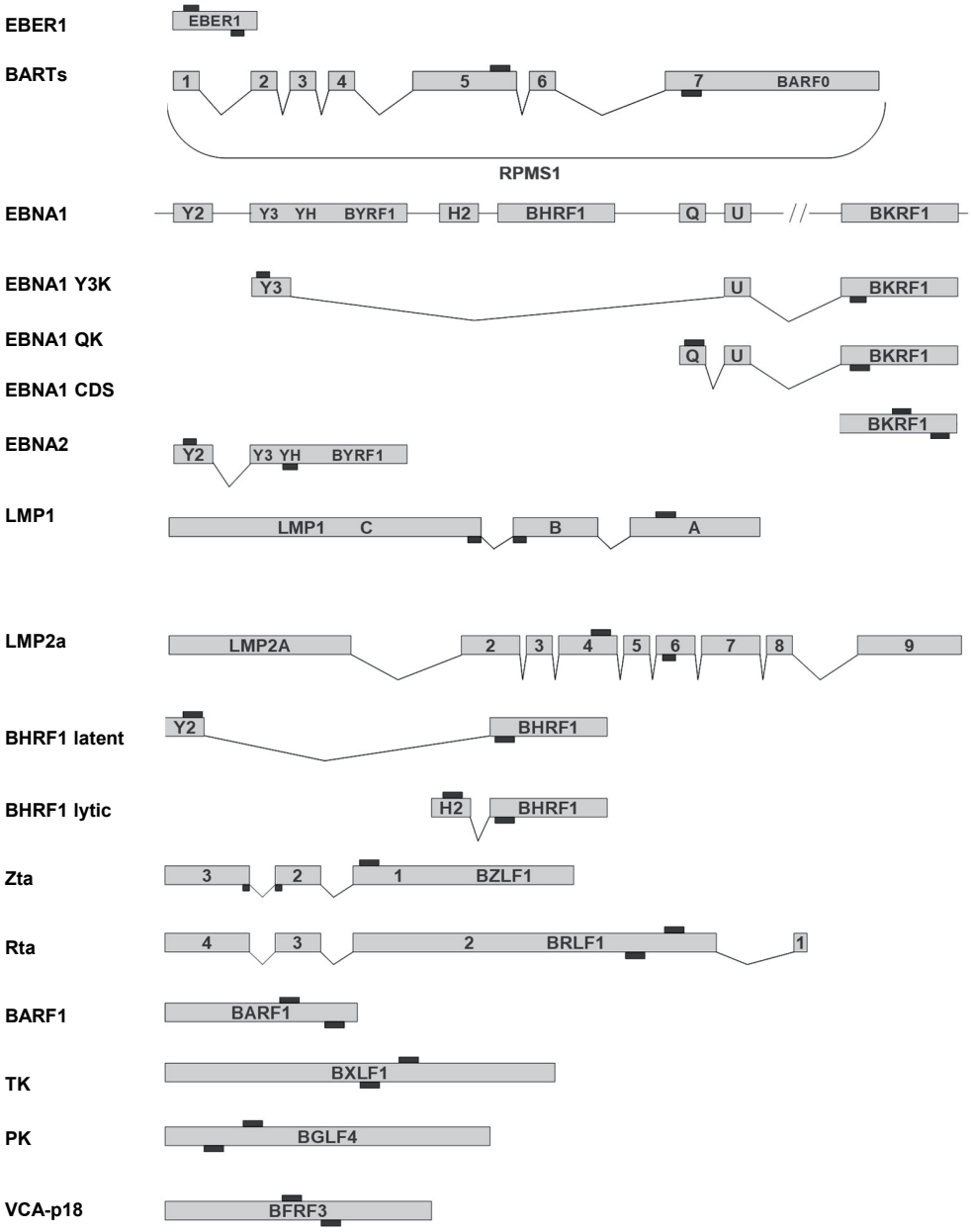

Figure 1. Schematic representation of the location of primers (solid small bars) used for the amplification of viral targets. Boxes represent the exons and t lines the combination of exons after splicing. The primer sequences are given in Table 1. The location of the Z1B forward primer is shifted 4 bp compared to the Z1 primer.

primers for viral genes and one cellular gene (Table 1, Figure 1), at 2 pmol/µL each in RNase free H_2O) to RNA pellet followed by incubation of 10 min at 65°C. Subsequently, cDNA was transcribed by AMV reverse transcriptase (Promega) during 1 h at 42°C.

cDNA was diluted at least 10 times before use in the PCR reaction. 2.5 µL cDNA was added to 7.5 µL mastermix containing SybrGreen Mastermix (Roche), and 10 pmol/µL forward and reverse primers (Table 1). Primer concentration for ZEBRA amplification needed to be reduced to 4 pmol/µL to avoid non-specific products in the PCR. Amplification was performed in a LightCycler 480 (LC480, Roche) with the following cycling conditions: 10 sec pre-incubation at 95°C, followed by 50 amplification cycles consisting each of denaturation at 95°C, annealing at 55°C and extension at 72°C followed by generation of the melting curve (Supplementary Figure S1 and S2).

After analysis of the melting curve for proper specificity of the products, quantification was performed. A target-specific standard curve was made using serial 10-fold dilutions (10^6–10^1 copies per PCR) of the plasmid pool containing all targets, which was amplified in parallel with the samples in the same plate. The second derivative method (LC480 software) was used for defining the cycle of positivity. The multi-plasmid standard curve was used to quantify the exact amount of RNA molecules in the cell lines. New plasmid stocks were calibrated against the original stock before use in order to ensure consistent quantification. A variation below 2-fold was accepted and corrected for.

Quantitative real time EBV-DNA PCR

EBV-DNA load and genomic contamination in cDNA were determined by quantitative real time PCR (LightCycler480, Roche) targeting a 99 bp segment within a highly conserved EBNA1 sequence, which is outside of the region transcribed during cDNA synthesis. The probe master mix used was supplemented with primer QP3 and QP4 and hybridization probes as described previously[39]. Real-time PCR reagents were obtained from Roche Diagnostics. 10-fold dilutions of spectrophotometrically quantified plasmid DNA containing the EBNA1 target sequence were used to create a standard curve. Quantification of the specific amplification products was performed with the second derivative software of the LC480 (Roche) using the standard curve based on human placenta DNA.

Results

Sensitivity and specificity of cDNA synthesis

To determine RNA profile in biological samples in one reaction we developed a multi-primed quantitative RT-PCR assay, which allows detection and quantification of 16 viral

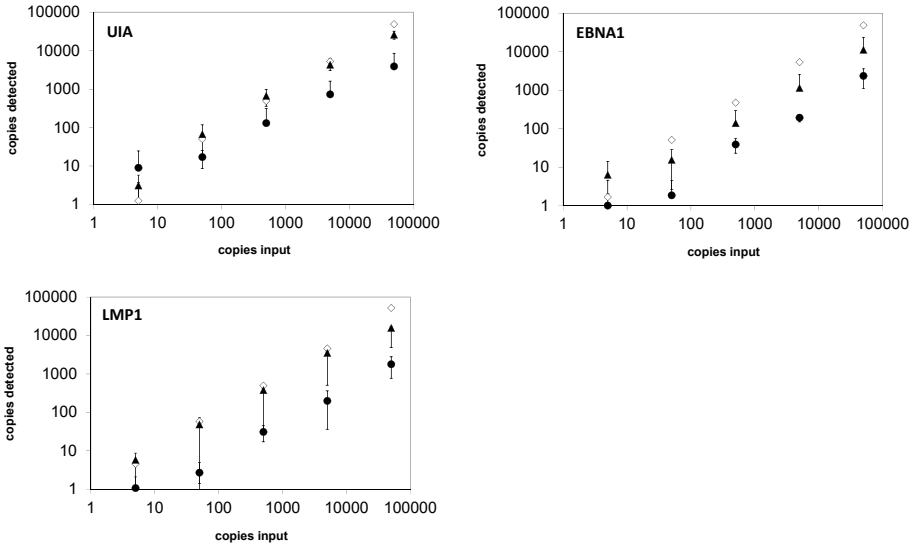

Figure 2. Performance of ROT versus plasmid pool. Since the stability of ROT is low, 2 concentrations of stocks for storage at –80°C were compared (10^{10}, triangle, 10^{8}, circle). The 10^{10} stock showed a high reproducibility. However for practical reasons a plasmid standard is preferred. Plasmids (square) as standard were compared to the performance of the ROT stocks. The standard deviation in this series is not visible due to low value. The plasmids showed a high reproducibility and revealed identical molecules as the RNA stock indicating for quantification ROT could be replaced by plasmid.

(Figure 1) and one cellular transcript. For ZEBRA, 2 types of primer combinations were used. In the EBV-positive carcinoma cell lines C666.1 and SNU719 and most clinical carcinoma samples a mutation is present located in the forward primer. Therefore a primer specific for carcinoma cells was developed (Z1B, Table 1).

To obtain maximal sensitivity a multi-primer mix containing reverse primers of all targets was used for synthesis of cDNA as the use of random hexamer primers proved to yield reduced efficiency of cDNA synthesis (Supplementary Figure S3). For most gene targets the results with specific primers were more consistent and showed a 2-50 fold higher yield compared to using random priming. This finding was reproducible over different LCL lines. cDNA synthesis using a multi-primed RT reaction proved highly efficient and reliable. Linearity of cDNA synthesis was reached for all targets allowing an accurate quantification (Figure 2).

Standards for quantification by multi-primed quantification RT-PCR

To determine the sensitivity of each of the primers a standard curve with known copy numbers must be generated. Initially, a mixture of gene-specific run-off transcripts (ROT) was used as standard for determining the concentration of RNA targets in the samples. ROT of all targets were quantified individually and then pooled to a concentration of

10^{10} of each target/µL. ROT-based standard curves gave accurate and reproducible results as shown in Figure 2.

However, to avoid the innate instability of ROT stocks, quantification of mRNA levels based on ROT was replaced by plasmids, which could be directly used in PCR amplification, revealed similar results in the whole dynamic range of the PCR (Figure 2). Therefore plasmids were used as a standard throughout. Plasmids containing amplicon sequences for all analysed genes were pooled ensuring that the standard curve was similar for all targets (master plasmid stock 10^{12} copies per µL, working plasmid stock 10^{8} copies per µL).

Quantification of single plasmid in dilutions of the pool proved identical in multi-primed PCR compared to the standard curve of the plasmid alone (Figure 3). Specificity of each RT-PCR product was checked by melting-curve analysis on the LC-480 instrument, in parallel with appropriate controls in each experiment, as detailed in supplementary Figure S1 and S2. New dilutions of the plasmid pool results were calibrated to the old standard and when needed adapted by calculation, which was never exceeding 2-fold corrections. This approach to quantify initial RNA derived cDNA in subsequent PCR using a plasmid pool for standard curve made it possible to calculate the number of RNA molecules per cell equivalent.

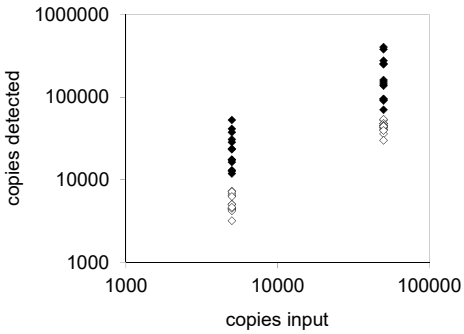

Figure 3. Performance of single plasmids as quantitative calibrators compared to the use of a plasmid pool containing 15 plasmids. The reproducibility of quantification of plasmids was similar although the level was slightly lower in the plasmid pool. The single plasmid (white) was used to validate the plasmid pool (black) and therefore a correction was made in the subsequent use of the plasmid pool.

For all targets the quantitative RT-PCR reached an analytical sensitivity between 5 to 50 copies of input, except for ZEBRA which had a detection limit of 500 copies RNA which is comparable to our prior published data[37] (similar data for both primer pairs, Table 2).

Table 2. Analytical sensitivity of multi-primed RT-PCR as defined by the percentage of a positive result of of 5, 50 and 500 plasmid copies input per PCR.

	Sensitivity (% positive)		
	500	50	5
EBER	100	100	0
BARTs	100	83	42
EBNA1Y3K	100	100	17
EBNA1QK	100	92	17
EBNA1CDS	100	100	100
LMP1	100	100	100
LMP2	100	100	100
EBNA2	100	100	67
BHRF1Y2	100	100	100
ZEBRA*	100	0	0
Rta	100	67	0
BARF1	100	100	75
BHRF1H2	100	50	25
TK	100	100	100
PK	100	100	42
VCAp18	100	100	100
U1A	100	75	0

The experiment was performed in 12 replicates.
*Both primer combinations of ZEBRA have identical sensitivity.

The reproducibility of the plasmid pool used for standardization was high, showing low standard deviation in the 3 separate experiments as presented in Figure 2. The relative sensitivity was determined by diluting JY cells in a background of 10,000 EBV negative BJAB cells as described previously[37]. For most targets a range of 1-100 cell equivalents could be readily detected (Table 3).

Validation of real time quantitative RT-PCR

To assure that the cDNA synthesis reaction is robust in that it performs with similar efficiency in independent reaction tubes, the RNA profile of a known sample of JY LCL cells (250 cells) was analysed in several repetitions and the results were consistent for all RNA targets (supplementary Figure S1 and S2). A common problem with DNase I treatment is that low genomic contamination cannot be completely excluded. However the EBV genomic contamination can be quantified precisely by EBV-DNA PCR, targeting a distinct and highly conserved region of the BKRF1 encoded EBNA1 gene, as described before[35].

Table 3. Relative sensitivity of multi-primed RT-PCR as defined by the number of EBV positive JY cells needed to detect a positive result in a background of 10,000 EBV negative BJAB cells. Transcripts of EBNA QK are not present (np) in JY cells, which was confirmed by the PCR results.

	number of JY cells
EBER	1
BARTs	10
E1Y3K	100
E1QK	np
E1CDS	1
EBNA2	1
LMP1	1
LMP2	100
BHRF1Y2	1
BHRF1H2	100
ZEBRA	1000
Rta	10
BARF1	100
PK	10
TK	10
VCAp18	100

The PCR region used for viral load determination is located beyond the reverse primer of EBNA1 CDS. Therefore the sequence of amplification in the viral load PCR is not transcribed into cDNA, allowing the quantification of genomic EBV-DNA in the background of the synthesized gene-specific cDNA. In DNase I treated cultured cell RNA samples the genomic contamination was generally very low and below 0.1%. Nevertheless, all data originating from intronless targets were corrected for the remaining amount of genomic EBV-DNA.

Specificity was confirmed by consistent negative results for EBV markers, but clear signals for SNRPA RNA in a diverse set of EBV negative cell lines: BJAB (supplementary Figure S1 and S2), AK31, Mutu 9, AGS, and HONE1 (data not shown).

Relative sensitivity of the RT-PCR was determined as the amount of detectable JY cells in the background of the negative BJAB cell line (Table 3). JY cells show a broad spectrum of gene expression originating from the latent cells and from cells showing spontaneous expression of lytic genes. This occurs in about 5% of the population as assessed by ZEBRA and EAd immunofluorescence staining[40].

Quantitative RNA levels of host and viral genes in Burkitt's lymphoma (BL) cell lines

The multiplex EBV qRT-PCR was used to analyse several EBV positive cell lines that originated from different epithelial and lymphoid malignancies. The first observation was that the cellular housekeeping gene SNRPA was expressed at different levels in the various cell lines (data not shown). Since the SNRPA mRNA expression level was high and reproducible per cell in cell lines tested, SNRPA transcript analysis could be used as a quality and loading control.

Latent BL cell lines Akata, Daudi, P3HR1 and HH514, showed expression of EBER, BARTs and Qp-driven EBNA1, with low copies more clearly detectable using the E1CDS primer set, confirming Latency I (Figure 4A). However low copy lytic gene expression was observed from the early genes BHRF1H2, BARF1, TK and PK as well as the late gene VCAp18 (mostly below an average of 1 copy per cell). This low expression level of lytic transcripts is consistent with the observation that a small population of cells in a culture population spontaneously reactivates into lytic cycle dependent on culture conditions. The Daudi, P3HR1 and HH514 cells are closely related latency type I Burkitt lines containing type-2 EBV genomes but lacking the EBNA2 gene, as confirmed by the absence of EBNA2 transcripts. P3HR1, similar to Daudi has known spontaneous lytic cells (<5%) in conventional culturing, in contrast to the HH514 derivative, which defined as a true latency type I.

Established activated BL cells lines Raji, Jijoye and Namalwa show a type III latency profile (Figure4B), including EBNA2, Cp/Wp driven EBNA1, EBERs, BARTs, LMP1, and LMP2. In these activated BL cells latent BHRF1 (BCL2 homologue) was detected as well. The Raji genome is deleted for genes involved in DNA replication (e.g. BALF2-5) and is therefore defective in expressing late lytic gene[41]. The Namalwa cell lines has two tandemly integrated viral genomes which allow only a restricted latency RNA profile to be expressed and LMP2 is absent due to disruption of the LMP2 reading frame by integration into the host genome[42]. The Jijoye (Figure 4B) and JC-5 (Figure 4C) showed similar RNA patterns[43]. These data in B cell lines show that the RNA profiles are in agreement with previous publications. Due to the high sensitivity of the RT-PCR additional lytic transcripts were detected in cells previously considered truly latent, confirming recent findings[31,33,35].

Quantitative viral RNA levels in EBV infected lymphoblastoid cell lines (LCL)

Established LCLs X50-7, JY, and RN are derived from EBV B95.8 infected peripheral blood derived B cells. The tightly latent IB4 cell line was derived from umbilical cord B cells. In all LCLs RNA patterns according to latency type III were observed as defined by high

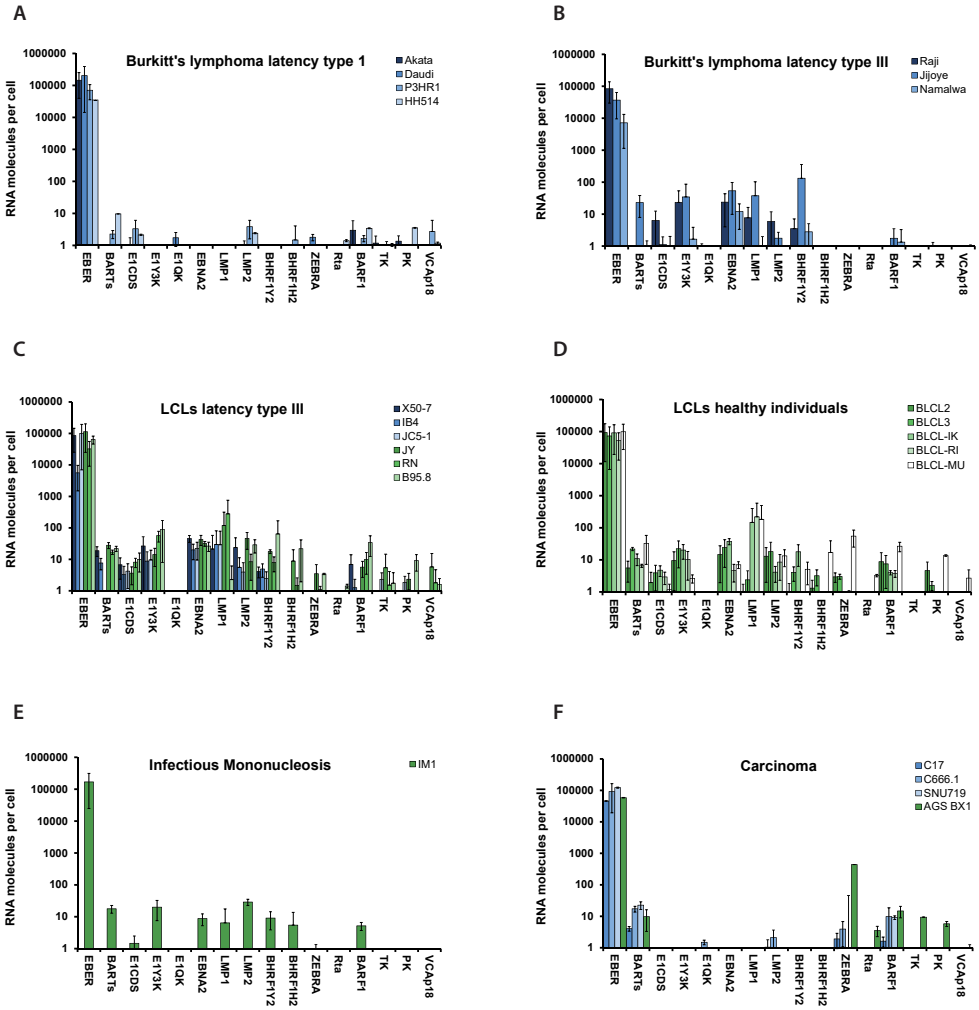

Figure 4. RNA profiles of 16 viral genes reflecting the different latency types and lytic cycle. Presented in n blue are cell lines considered to be latent, in green cell lines which have a small population of cells with reactivated EBV. For these analysis multi-primed cDNA of 1250 cells were used as input for PCR, except for the high copy genes U1A and EBER. A, Burkitt's lymphoma cell lines with a latency type I. B, Burkitt's lymphoma cell lines with a latency type III. C, Low passage B95.8 transformed BLCL lines (BCL 2 and 3) and the LCL cell lines, which were obtained by spontaneous outrgrowth of B cells from the blood of healthy individuals, wt-BLCL–IK, –RI and –MU containing endogeneous EBV from donor origin[45]. D, Established high passage LCL cell lines transformed by the B95.8 strain of EBV. E, IM1 cell line produced from a patient with Infectious Mononucleosis. F, carcinoma cell lines of which C666.1 and C17 are NPC cell lines naturally infected by EBV (C17 passaged in mice); SNU719 is a GC cell line naturally infected by EBV; AGS-BX1 is a GC cell line infected with a recombinant EBV.

level expression of EBERs, BARTs, Cp/Wp derived EBNA1, EBNA2, LMP1 and LMP2 as well as the latent BHRF1 (Figure 4C).

JY and RN cells showed relatively high expression of lytic transcripts, including BARF1, most likely reflecting that about 3–5% of these cells spontaneously enter the lytic cycle. LMP2 is expressed by IB4 although this has an integrated EBV genome like Namalwa cells. However one of the four integrated genomes contains an intact LMP2 gene, since the viral genome was linearized by a break after the LMP2 gene instead of the terminal repeat region[44].

An RNA profile was also obtained for low-passage LCLs from two healthy individuals infected with the B95.8 EBV strain. The expression of the viral genes showed a similar pattern as seen in B95.8 LCLs as described above (Figure 4C, 4D). However LMP1 expression was remarkably reduced. In contrast, wt-LCLs obtained by spontaneous outgrowth directly from the blood of healthy individuals showed high LMP1 expression (Figure 4D)[45].

The spontaneous wt-LCLs showed a latency type III pattern expressing the EBNA1, EBNA2, LMP1 and LMP2 besides EBER and BART transcripts. BARF1 mRNA was also detected in most LCL lines. The low passage wt-cell line IM1 obtained by direct B cell culture from a patient with acute IM showed a highly active transcription of viral genes (Figure 4E). Besides the noncoding EBER and BARTs transcripts, mRNA encoding the latent genes EBNA1, EBNA2, LMP1, LMP2, BHRF1 and BARF1 was readily detectable. Importantly, all LCL-like lines lacked Qp-driven EBNA1, but revealed clear Cp-driven EBNA1 transcription, important for maintaining the viral genome in rapidly growing cells.

Quantitative RNA levels of viral genes in EBV positive carcinoma cell lines

RNA profiling of nasopharyngeal and gastric carcinoma cell lines showed a high EBER content (Figure 4F). The transcriptional activity of other latent genes is lower compared to the lymphoid cell lines and EBNA2 was consistently negative confirming the latency type II characteristic for these carcinoma lines. BART transcripts are readily detected in all cell lines, but the other latent genes were expressed at very low.

Only in C666.1 the EBNA1 RNA could be detected. LMP1 is detected in C17 and absent in C666.1 cells. LMP1 is also absent in the gastric carcinoma cell lines as expected. The BARF1 transcript is present in the NPC and gastric cell lines. The AGS-BX1 cell line has spontaneous lytic activity indicated by the presence of ZEBRA, BHRF1, RTA and TK transcripts. The other carcinoma cell lines, C17 and SNU 719, have a more restricted latent profile with only sporadically detection of some lytic RNA.

Transcriptional levels in chemically induced Burkitt's lymphoma and carcinoma cells

Several LCLs showed low (not higher than 10 copies per cell) but defined lytic cycle mRNA signals reflecting spontaneous virus reactivation. Although the overall level of lytic transcripts per cell might be low, a small portion of cells with high numbers of lytic RNA molecules (Figure 4B-E) may be responsible for this. The highly latent HH514 cell line was induced for lytic gene expression by TPA and butyric acid and after 24 h the viral RNA profile was analysed (Figure 5A). At each time point cells were counted and RNA copies were related to the number of viable cells.

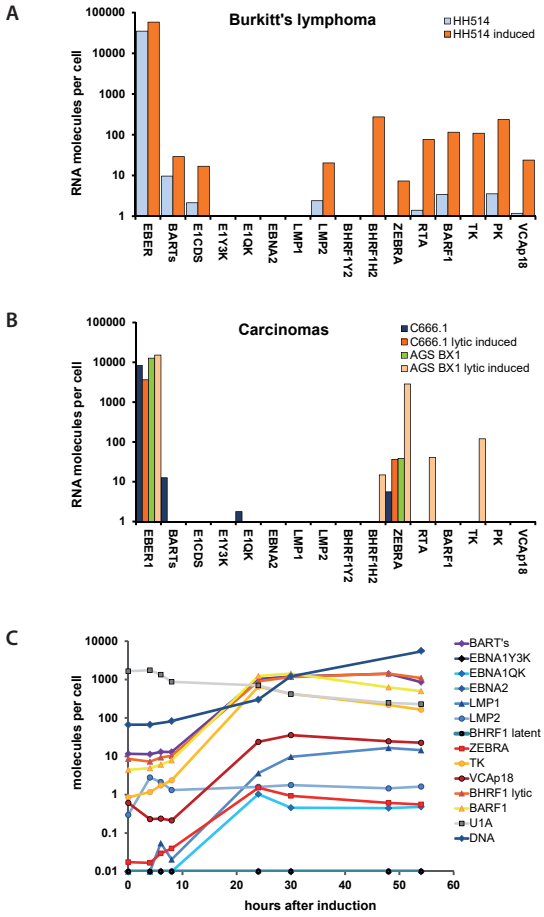

Figure 5. A, RNA profile of the Burkitt's lymphoma cell line HH514, presented in blue is the cell line in latency, in orange the cell line after 24 h induction with TPA and butyric acid. B, RNA profile of the carcinoma cell lines, C666.1 (in blue) and AGS-BX1 (in green) in latency; C666.1 (in orange) and AGS-BX1 (in light orange) after 24 h induction with 3 μM GCb and 0.3 mM VPA. C, Time course after induction of the lytic cycle of Burkitt's lymphoma cell line HH514.

The latent genes EBNA2 and LMP1 were not induced whereas EBNA1 and LMP2 were 4–7-fold induced together with the immediate early gene ZEBRA. A 100–1000-fold increase in early genes PK, TK and BHRF1 was found. Lytic induction resulted in a rise of the lytic transcripts between 8 and 24 h, whereafter most RNA levels remained constant (Figure 5B). The increase in viral DNA was observed after 20 h at the time point that the early phase viral DNA polymerase (BALF5) allows new viral genome replication, which parallels the detection of the late transcript VCAp18 (BFRF3). The carcinoma cell lines C666.1 and AGS-BX1 were induced for 48 h with 3 μM GCb and 0.3 mM VPA[22,23].

Identical to the latent genes, the expression level of the lytic genes was very low (less than 1 copy per cell) in the C666.1 cell (Figure 5C), with the exception of the ZEBRA transcript which was induced above 10 copies per cell. In AGS-BX1, known to be more sentitive to lytic induction, higher levels of the lytic gene expression were reached although the late VCAp18 transcripts could not be detected.

Discussion

For quantification of specific RNA transcript levels real-time quantitative RT-PCR has become the method of choice. Standardization and accurate quantification of RNA levels against a molecular defined calibrator is advantageous compared to commonly used relative quantification against cell line RNA extracts. The quantification method described here uses plasmid-based standard curves for each target gene to determine the amount of target transcripts per cell.

We developed a multi-primed quantitative real-time RT-PCR for 16 viral and one cellular gene aiming at simultaneously assessing different EBV latency programs and possible lytic cycle gene expression. This quantitative RT-PCR not only revealed the expected latency profiles in EBV associated lymphoma and carcinoma cell lines, but showed unexpected lytic transcripts present at low levels, confirming recent findings by others using RNAseq or multiplex array analysis[29,31,33].

The plasmid pool based quantification of cDNA proved superior to standardization with artificially synthesized RNA. A plasmid pool of the different targets was created to have high flexibility in the transcription profile to be analyzed. The use of a single multiple gene plasmid is however preferred for fixed gene analysis[35]. Gene specific primers for the cDNA synthesis, rather than random priming of cDNA synthesis produced the highest sensitivity. Especially for short RNAs (EBERs) the random hexamer priming resulted in a considerable decrease in cDNA synthesized, confirming gene specific priming as

method of choice[46]. All reverse primers of the target genes were combined in a multi-primed cDNA synthesis reaction. The real time quantitative RT-PCR showed a very high sensitivity and specificity for all targets except one, i.e. ZEBRA.

The high sensitivity of this RT-PCR raises the question what the relevance is of transcript levels of 1–10 copies per cell. Although with high input of cells the quantification is robust, the biological relevance might not be high because these transcripts might not be evenly distributed throughout the cell population, but present in a minority subpopulation. It is well known that many latent EBV infected cell lines in bulk cultures have some level of lytic (abortive) reactivating cells triggered by and dependent on cell culture conditions. To address the single cell expression levels an alternative approach would be recommended using this multi-target quantitative RT-PCR in a limiting dilution or cell sorting set-up[30].

Analyses of several EBV positive lymphoma and carcinoma cell lines resulted in the expected RNA profiles, accordingly to their latency program and the presence of lytic cells. This EBV specific quantitative RT-PCR is suitable for obtaining RNA profiles both in cell lines and patient materials, as recently demonstrated by us[22,23].

In the cell lines the cellular housekeeping gene SNRPA can be used for the normalisation of viral gene expression. However in patient material, where tumor and nontumor cells will occur in variable percentages, a use of EBV-encoded gene might be more appropriate for normalization. Overall the viral EBNA1 gene had steady, but low expression (till 10 copies/cell) enabling to use this gene as standard for normalised quantification of the EBV content in patient material from EBV associated malignancies. A similar constant EBNA1 expression level was observed in previous studies with in different BL cell lines analysed by RT-PCR and micro array techniques[28].

For normalisation of (patient) samples the coding region of EBNA1 (EBNA CDS) could be used, whereas in understanding of EBV regulation the analyses of the untranslated region of EBNA1 might be more informative (EBNA1 Y3K and EBNA QK). In majority of cell lines, the level of EBNA1 RNA was very low, which is in agreement with results of Tierney et al.[35]. The low abundance might be related to the high EBNA1 protein stability with a 36–48 h half-life[47]. In addition, the abundance of EBNA1 transcript is cell-cycle dependent with the highest expression level reached in dividing cells at the S phase[47].

Alternatively, the constant presence and elevated expression levels of the noncoding BART RNA transcript suggest this to provide a more robust marker for normalisation. Normalisation can also be done by using the EBV-DNA copy number in the initial

sample or lysate, which would be a useful parameter when the number of infected cells is unknown.

The analysis of well-known latency type I BL-derived cell lines showed high expression of EBER with low expression of BART RNA and Qp-driven EBNA1 and LMP2 mRNAs, and variable low level expression of some lytic genes (Figure 4A). LMP1 mRNA was not detectable in Daudi and P3HR1, confirming prior studies[48]. The BL cell lines known to maintain latency type III (Fig 4B) showed presence of C/W promotor driven EBNA1 and EBNA2 transcripts besides additional latent genes LMP2 and BHRF1. In Namalwa, LMP1 expression was below detection limit[28,49].

Latency III patterns were observed in LCL transformed with the B95.8 strain of EBV. EBNA1 expressed by the C/W promoter, LMP1, LMP2 and EBNA2 were highly expressed. The cell lines JY and RN showed presence of lytic transcripts indicating a small cell population harbouring reactivated EBV. In B95.8 transformed LCLs of PBMCs from different donors, EBNA1, LMP1 and LMP2 were detectable, with LMP1 showing the highest expression levels. Wt-LCLs generated by spontaneous B cell outgrowth displayed a higher transcriptional level of the latent genes as well as the activity of many lytic cycle specific genes.

Several cell lines showed spontaneous lytic cycle induction as indicated by presence of ZEBRA, RTA, TK, PK and VCAp18 RNA at low levels. However these lytic transcripts are produced by only less than 5% of the cells, therefore RNA expression per cell might be even 20 times higher. In our analysis of lytic induced B cells (HH514) by TPA and butyric acid a steep increase was observed for all lytic transcripts after 24 h of induction. Next to the well-known transactivator ZEBRA, also the early (PK, TK) and late (VCAp18) transcripts were detected in high numbers per cell equivalent. A rapid rise of transcripts from all the phases of the replicative cycle was detected after 16 h. The presence of late transcripts was unexpected, but appears in agreement with recent data in Akata cells induced by B cell receptor cross-linking[35] indicating regulatory mechanisms for the delayed expression into protein or abortive lytic gene expression[35,50].

The analysis of cultured cells containing sporadic spontaneous lytic cells reveals the complexity of RNA quantification in cell equivalents which may result in misinterpretation due to cell heterogenicity. Therefore a novel method is needed in order to acquire the RNA profile per cell, such as single cell RT-PCR. A combination of the method described by Hochberg and Thorley-Lawson[30] and the multi-primed RT-PCR could provide a more accurate measurement of the transcript level per cell[50].

The multi-primed quantitative RT-PCR designed for the quantification of RNA molecules per cell could be used to standardize results obtained in several labs and contribute to the understanding of EBV infection, since it offers a high sensitivity and specificity. The detailed analysis could potentially lead to new diagnostic approaches and could be useful in monitoring therapy responses. In a recent clinical trial we demonstrated the use of this quantitative RT-PCR approach for measuring EBV lytic gene expression in vivo in NPC patients undergoing a novel virus reactivation therapy[25].

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Supplementary

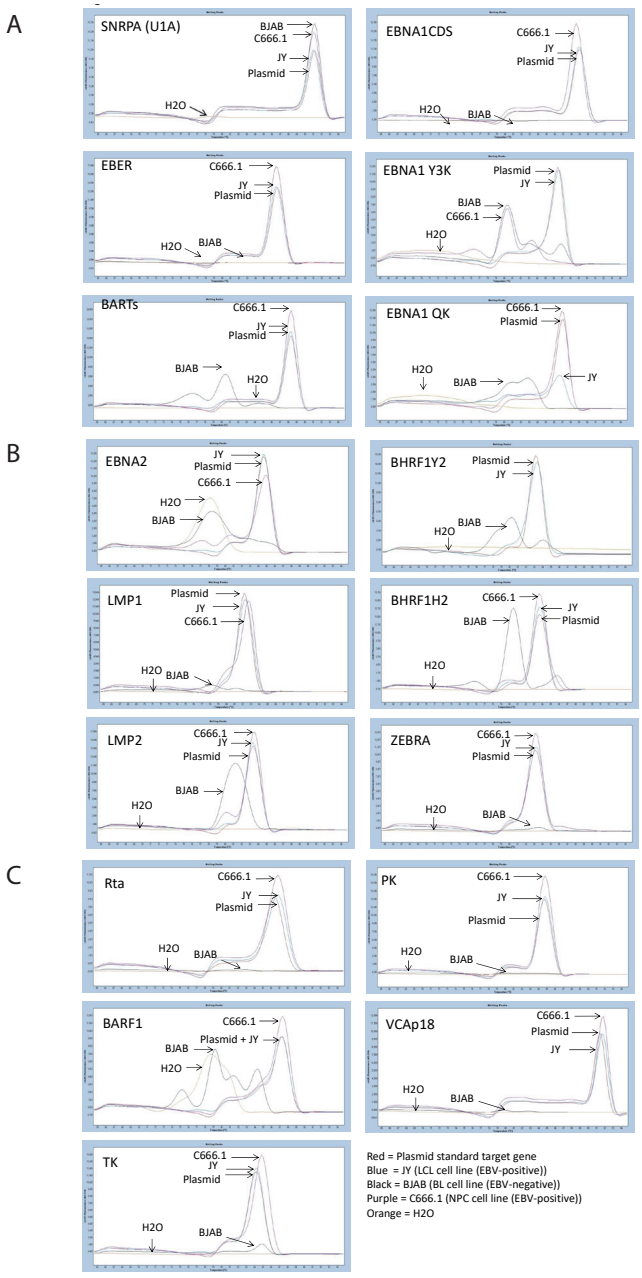

Figure S1. Melting curve analysis of RT-PCR products generated for all 17 individual EBV mRNA targets, using RNA extracted from EBV-positive cell lines (JY LCL; C666.1 NPC), EBV-negative line (BJAB), in addition to the multi-target plasmid pool as positive control and H2O as negative control. A, B, C, Melting curve analysis for SNRPA (U1A) and all EBV mRNA targets in JY, C666.1 and controls.

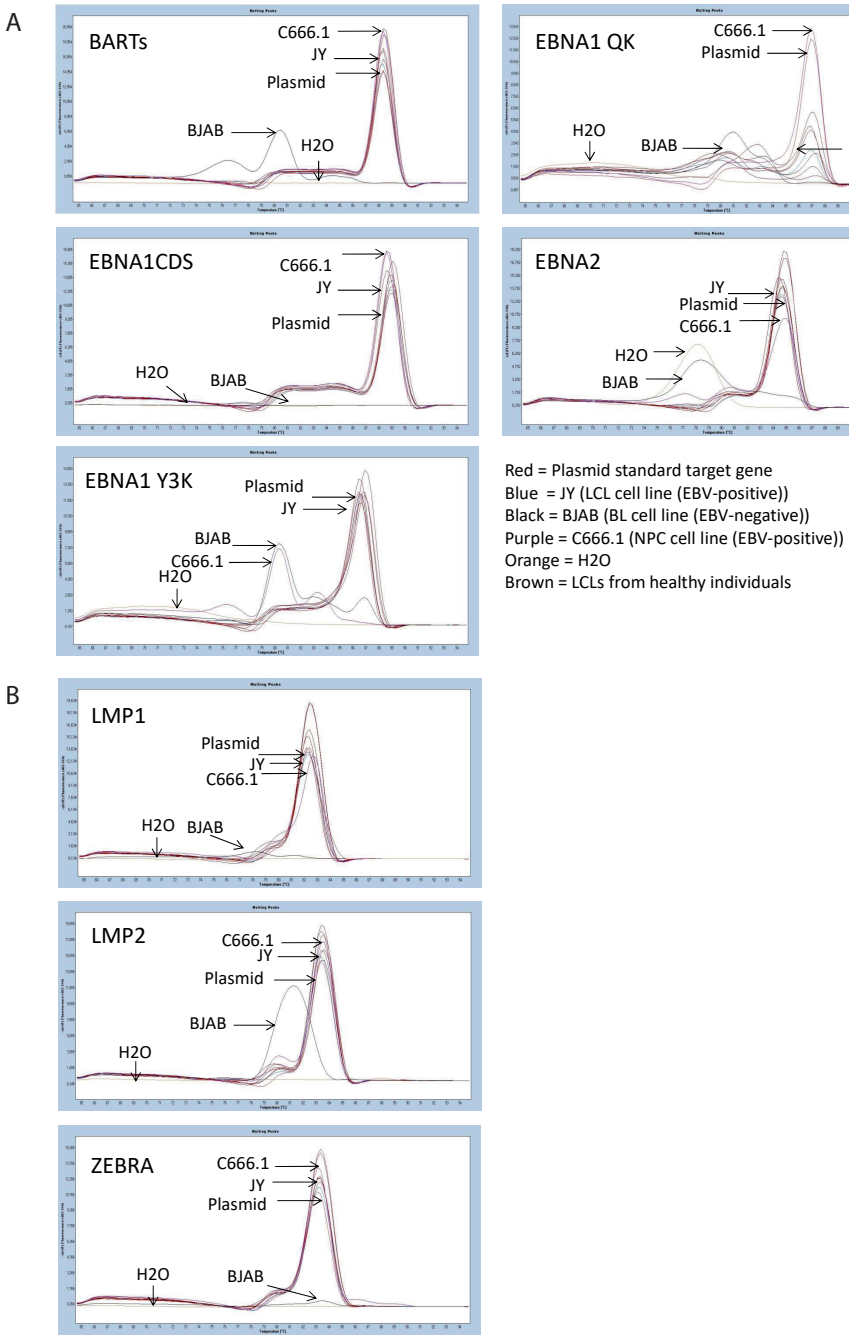

Figure S2. Melting curve analysis of RT-PCR products generated for selected EBV mRNA targets, using RNA extracted from six different EBV-positive LCL's, in addition to the cell lines and controls indicated in supplementary Figure S1A-C. A, B, Different EBV mRNA targets with spontaneous wtLCLs (brown) and controls.

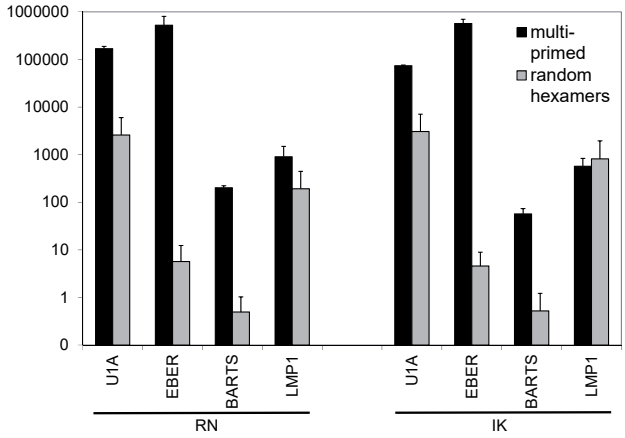

Figure S3. Difference efficiency of cDNA synthesis using either gene specific primers (multi-primed) or random hexamers. The black bars represent gene specific cDNA synthesis and gray bars use of random hexamers in the graph representing two independent experimental replicates. RN and IK are LCL lines created by B95-8 transformation and spontaneous outgrowth, respectively.

6

Curcumin analogs as putative Epstein-Barr virus lytic activators for adjuvant treatment in carcinomas

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Submitted

Abstract

Introduction: Epstein-Barr virus (EBV) persists in nasopharyngeal (NPC) and gastric carcinomas (EBVaGC) in a tightly latent form. Cytolytic virus activation (CLVA) therapy employs gemcitabine and valproic acid (GCb+VPA) to reactivate latent EBV into lytic phase and antiviral valganciclovir to enhance cell death and prevent virus production. CLVA has proven safe with promising clinical responses in patients with recurrent NPC. However a major challenge is to maximize EBV lytic reactivation by CLVA. Curcumin, dietary spice used in Asian countries is known for its antitumor property and therapeutic potential. Novel curcuminoids which were developed to increase efficacy and bioavailability may serve as oral CLVA adjuvant.

Methods: We investigated the potential of curcumin and its analogs to trigger the EBV lytic cycle in EBVaGC and NPC cells. EBV-reactivating effects were measured by immunoblot and immunofluorescence using monoclonal antibodies specific for EBV lytic proteins.

Results: Two of the hit compounds (**41**, **EF24**) with high lytic inducing activity were further studied for their synergistic or antagonistic effects when combined with GCb+VPA and analysed by cytotoxicity and RNA profiling assays to measure the EBV reactivation. Curcuminoid as a single agent significantly induced EBV reactivation in recombinant GC and NPC lines. The drug effects were dose- and time-dependent.

Conclusions: Micromolar concentration of curcuminoid **EF24** enhanced the CLVA effect in all cell systems except SNU719, a naturally infected EBVaGC cells which carry a more tightly latent viral genome. These findings indicated that **EF24** has potential as EBV lytic activator and may serve as adjuvant in CLVA treatment.

Introduction

Epithelial malignancies associated with Epstein-Barr virus (EBV) infection include undifferentiated nasopharyngeal carcinoma (NPC) and a subset of gastric carcinoma (GC)[1]. EBV is detected in most of NPC cases in endemic countries. More than 80% of post-surgical gastric stump or remnants and the lymphoepithelioma-like (LEL) GC and about 10% of gastric adenocarcinoma cases around the world show evidence of EBV infection, called EBV-associated gastric carcinoma (EBVaGC). Overall, patients with EBVaGC had longer survival than those GC patients with no evidence of EBV[2,3]. In NPC both hyperplastic, preinvasive and invasive lesions of the nasopharynx show the presence of monoclonal viral episomes[1], whereas EBV genomes persist only in fully malignant EBVaGC lesions in the proximal stomach[2,3]. Contrary to EBVaGC which occurs worldwide, NPC is rare in most parts of the world, but notoriously common in the Southern China, Southeast Asia, North America and the Mediterranean region[1,2].

Most EBV-associated carcinomas contain viral DNA that perists in a latent state with only a limited set of viral genes being expressed. EBV nuclear antigen 1 (EBNA1), small RNAs EBER1 and 2, BARF1 protein and *Bam*HI-A rightward transcripts (BART) encoding 40 microRNAs (miRNAs) are expressed in all carcinoma cells[1]. EBVaGC, however has a unique, modified latency type I expression pattern with a variable expression of LMP2A, but no LMP1. In contrast, 50–80% of NPC cases express LMP1 in addition to LMP2A which characterizes the NPC latency pattern as type II[2].

Besides personalized T-cell based immunotherapy, to date no effective virus-targeted treatment has been developed for NPC and EBVaGC[4,5]. Recently, chemical EBV reactivation followed by administration of an antiviral cytolytic drug was proposed for the treatment of EBV-positive lymphomas and carcinomas refractory to conventional chemotherapy or radiation[4-7]. A recent clinical trial on cytolytic virus activation (CLVA) therapy using a combination of gemcitabine (GCb), valproic acid (VPA) and ganciclovir (GCV) demonstrated virus reactivation in vivo associating with promising clinical responses in end-stage NPC patients[6,7]. To ensure the selective killing of EBV-positive tumors, effective initiation of EBV lytic cycle and expression of viral kinases are required. These kinases phosphorylate nucleoside analogues (e.g. acyclovir, (val)ganciclovir; GCV) into their active forms which cause DNA chain termination leading to lysis of tumor cells and curtailing the release of infectious viral particles[4,8].

The mechanisms of induction of EBV lytic replication from latency using chemical inducers such as histone deacetylase inhibitors (HDACi), DNA demethylating agents and chemotherapeutic drugs have been extensively investigated[4,8,9]. Induction of

host cell differentiation and inhibition of NF-kB pathways are common themes that trigger EBV reactivation in latently infected cells[9]. Most existing chemical activators of EBV lytic cycle tested in clinical trials are associated with significant toxicities and restricted bioavailabilities[8,9]. Recent studies have identified various novel small organic compounds for potential EBV lytic inducers[9-11]. Whether these compounds will be tolerated in clinical settings remains to be determined.

Meanwhile, several human clinical trials have reported that curcumin, a polyphenolic compound known derived from *Curcuma longa* possess various therapeutic properties including anti-oxidant, analgesic, anti-inflammatory and anti-cancer activities due to its effect on multiple biological pathways including the inhibition of NF-kB[12,13]. It is a common ingredient in Chinese and Indian Ayurvedic medicinal recipes[12]. Importantly, curcumin is "generally recognized as safe" by the U.S. Food and Drug Administration (FDA), and used as adjuvant in approved cancer therapies[12-14].

Curcumin and its derivatives (known as curcuminoids) used alone or in combination with other drugs, increase cell death in a wide variety of tumor cells with minimal cytotoxicity[13-15]. Several curcuminoids have been developed to specifically address the pharmacokinetic limitations (poor oral bioavailability, rapid metabolism) of curcumin[15-20]. Curcumin and novel curcuminoids have recently been shown to limit the growth of NPC and GC cells in vitro and in a mouse tumor model, but without addressing the role of EBV in these tumors[13,15,20].

The central conjugated β-diketone linker in curcumin has been identified as a structural liability, contributing to its chemical and metabolic instability[17]. Replacing the conjugated linker with a monocarbonyl cross-conjugated dienone that is embedded within a ring structure has been widely employed as a viable solution. In this report, we explored various structural types that embodied this modification[16,17]. Curcuminoids with five different ring structures were investigated in this study[16-19], namely cyclopentanones **PGV-0, PGV-1, PGV-5, THPGV-0**, cyclohexanone **206**, piperidinone **EF24**, thiopyranones **211, 219** and thiopyranone dioxides **41, 227** (Figure 1).

The cyclopentanones were obtained from the UGM-VU collection of curcuminoids and two members (**PGV-0, PGV-1**) have been reported to possess cytotoxic, antiproliferative and anti-angiogenesis properties in tumor cells[18,19]. The piperidinone **EF24,** a widely investigated curcuminoid, has pleiotropic effects on inflammatory and oncogenic signaling pathways[20]. Like the cyclohexanones, thiopyranones and thiopyranone dioxides, **EF24** induced apoptosis in leukemic cells[16]. They were also more potent than curcumin, with the exception of the cyclohexanone **206** and thiopyranone **211**[17]. The

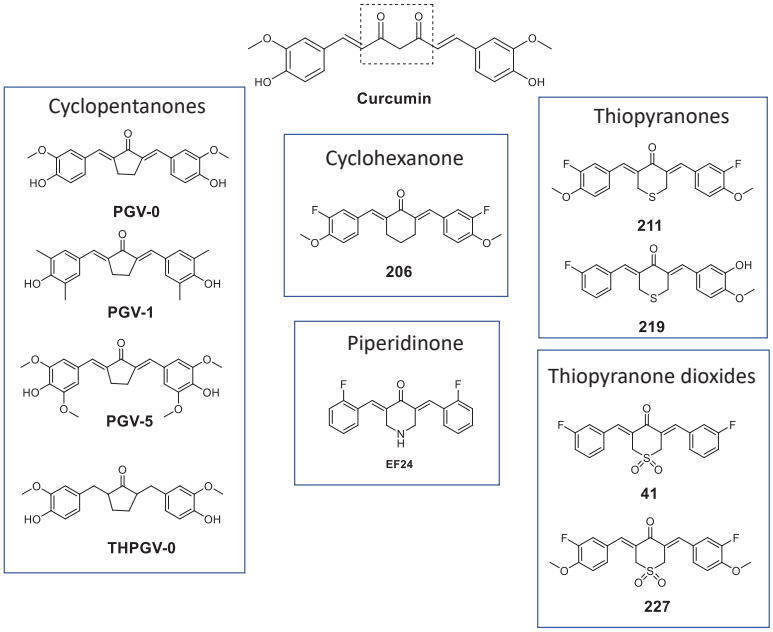

Figure 1. Novel curcuminoids through structural modification of curcumin to improve uptake. Curcumin structure and modifications of curcumin at its β-diketone linker and terminal phenyl rings to improve stability, bioavailability and pharmacokinetic profile as described in the materials and methods section.

most potent analogs were **41** > **227** > **EF24**, based on cell-based growth inhibitory concentrations (IC_{50}). The apoptotic effects of **41** and **227** were attributed to activation of the unfolded protein response in response to heightened endoplasmic reticulum (ER) stress induced by these compounds. The shortlisting of these compounds in preference to the others, aptly concurred with earlier studies which acknowledged their potencies in other unrelated investigations[16,17].

It is reported that reactivation of the latent viral genome in EBV-associated cancers can cause cancer cell death[21-23]. Due to the need of a highly efficacious targeted therapy with lower toxicity and preferably oral availability, an investigation into the potential of curcuminoids for initiating EBV reactivation in the context of CLVA therapy is timely. Here, we screen and identify the EBV lytic induction potential of curcuminoids used as single agent or as adjuvant to CLVA therapy in EBV-associated carcinoma cells. Regarding the relevance of cellular background to the EBV lytic inducers, we also confirm the cells' ability to express EBV lytic genes induced by newly identified activators in multiple NPC and EBVaGC cell lines. These curcuminoids are structurally distinct (Figure 1) and synergize with CLVA regimen to activate the lytic life cycle in latently infected cells while maintaining low toxicity.

Material and methods

Cell lines

EBV-positive GC cell lines (AGS-BX1, SNU-719) and EBV-positive NPC cell lines (HONE1-EBV, C666.1) were used in this study. AGS-BX1 harbours a recombinant EBV genome with an insertion of the neomycin-resistance gene and the green fluorescent protein (GFP) gene that disrupts the TK gene (kindly provided by Prof. em. L. Hutt-Fletcher[6]). HONE1-EBV was generated by introducing a green fluorescent protein (GFP) open reading frame in the recombinant Akata EBV genome into the EBV-negative NPC cell line HONE1 (gift from Prof. GSW Tsao[21]). EBV-negative GC (AGS) and EBV-negative NPC (HONE1) parental cell lines were used as negative controls. AGS, HONE1 and HONE1-EBV cells were maintained as detailed by Hui et al[24]. Natural EBV genome-carrying SNU-719 cells (purchased from the Korean Cell Line Bank, Seoul, Korea), natural EBV genome-carrying C666.1 cells and recombinant AGS-BX1 cells were cultured as described before[6,11,24,25].

Chemicals, plasmids, and antibodies

Natural purified curcumin was purchased from Sigma (Sigma-Aldrich), whereas novel analogs curcumin compounds were kindly provided by Dr. Mei Lin Go at The National University of Singapore (NUS, Singapore) and by Dr. Ritmaleni at The University of Gadjah Mada (UGM, Indonesia) in collaboration with Prof. em. Henk Timmerman of the Vrije Universiteit Amsterdam (UGM-VU, The Netherlands). The chemical structure of curcuminoids is summarized in Figure 1. The NUS collection consists of curcuminoids with the replacement of β-diketone by cyclohexanone (compound **206**), thiopyranone (compound **211**, **219**), thiopyranone dioxide (compound **41**, **227**) and piperidinone (**EF24**) moieties[16]. NUS compounds acted as activators of endoplasmic reticulum (ER) stress signaling pathways and apoptotic cell death in leukemic cells[17]. PentaGamaVunon (PGV) is novel curcuminoid from UGM-VU collection named benzylidenecyclopentanone. In PGV, the methylene and carbonyl groups have been omitted to produce more stable and potent compounds retaining anti-oxidant and anti-inflamatory activities. Two benzylidenecyclopentanone derivatives (**PGV0** and **PGV1**) demonstrated cytotoxic, anti-proliferative and anti-angiogenesis properties in tumor cells[18,19].

Suberoylanilide hydroxamic acid (SAHA), sodium butyrate (SB), gemcitabine (GCb) and valproic acid (VPA) (Sigma-Aldrich) were used as positive lytic activators. In all experiments 1% DMSO in culture medium was used as negative control. Two antibodies was used for immunofluorescence and immunoblot analysis; the BZ1 antibody for the detection of the lytic-switch protein BZLF1 or Zebra (kind gift from by Paul Farrell, Imperial College London, UK[6]) and the OT14E antibody for the detection of the BMRF1 or EA-D protein[26]. Rabbit antibodies against active caspase 3 and PARP-1/p89

(Promega) were used for detecting apoptosis-related proteins, respectively. A plasmid pool representing each EBV lytic target gene used for RT-qPCR messengerRNA (mRNA) quantification was detailed previously[27].

Cytotoxicity and cell viability assays

The cytotoxicity of hit compounds was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, the Netherlands) proliferation assay. Briefly, EBV-negative and -positive NPC and GC cells (2-5 x 10^4 cells) were seeded in 96-well culture plates without drug and allowed to adhere for 24 h[6,24]. Subsequently, t=0 was measured using MTT and hit compounds (**41**, **EF24**) including positive lytic activators SAHA and GCb+VPA[6,24] were added at different concentrations (in 2- or 5-fold). After 72 h, cells were lysed in DMSO and absorbance was determined at 540 nm in a plate reader.

The synergistic killing effect of hit compounds (**41**, **EF24**) and GCb+VPA was determined by trypan blue exclusion assay. EBV-positive GC and NPC cells grown to 70% confluence were treated with combination of hit compound (**41**, **EF24**) and GCb+VPA or GCb+VPA alone for 48-96 h depending on cell types (Figure 2A). Treatment with 5 μM SAHA or 3 mM SB was used as positive control[24]. Results are presented as percentages of viable cell populations among treated cells compared with those of untreated (DMSO) control. Experiments were repeated three times independently.

Immunoblot analysis

To analyze the expression of EBV lytic proteins, NPC and GC cells were treated with low (10 nm) and high (1.25 μM) concentrations of curcuminoids for 48-96 h (Figure 2A). Treatments with three activators at their lytic concentrations (3 μmol/L GCb + 0.3 mmol/L VPA, 5 μM SAHA, 30 mM SB) were included in the immunoblot analysis[6,24]. After treatment, the cells were pelleted and washed once with PBS. Proteins from the cell pellets were extracted and immunoblot analysis to detect Zebra and EA-D lytic proteins were performed as described previously[6,26,28].

FACS analysis, immunofluorescence assay and measurement of percentage of cells induced into lytic cycle

FACS analysis was used to screen lytic inducing capacity of various curcuminoids at nanomolar concentrations. GFP intensity representing induced lytic cells was measured by FACSCalibur Flow Cytometer (BD Biosciences). A gate was set to determine the percentage of lytic cells by analysing the GFP intensity of uninduced AGS-BX1 cells and after the CLVA treatment (GCb+VPA). Expression of GFP in the infected AGS-BX1 and HONE1-EBV cells following curcuminoid treatments visualized and counted under a fluorescence microscope (Leica).

Immunofluorescence staining was performed to analyse EBV reactivation in naturally infected GC (SNU-719) and NPC (C666.1) lines. Cells grown on cover slips coated with 0.1% gelatin were treated with either 1.25 μM hit compounds for 72-96 h. Treatment with SAHA and GCb+VPA were included as controls. Cells were fixed with cold methanol-acetone for 10 min and dried at room temperature. Subsequently, nonspecific binding was blocked by incubation in PBS containing 1% FCS. Glass slides were incubated with either Zebra (1:100) or EA-D (1:1000) antibodies in PBS-1%FCS for 60 min at room temperature. After washing with PBS containing 0.05% Tween-20, incubated with the secondary antibody conjugated with fluorescein isothiocyanate (FITC) for 30 min, washed again and slides were mounted in Vectashield (Vector Lab Inc.) containing 0.3 μmol/L 4′,6-diamidino-2-phenylindole (DAPI, Roche).

Incubation with PBS-1%FCS but no primary antibody and then with secondary antibody was used as a negative control. Staining patterns were observed under a fluorescence microscope. To quantify GFP-reactive AGS-BX1 and HONE1-EBV and FITC-positive C666.1 and SNU-719 cells, the percentage of positive cells was estimated by counting at least 500 cells from 5 high power fields. Amount of positive populations of AGS-BX1 and HONE1-EBV cells were then substracted from those of (GFP-positive) untreated (DMSO) control.

Quantitative RT-PCR assay for the detection of specific mRNA related to EBV lytic reactivation

To quantify viral lytic mRNA expression during the induction of EBV lytic cycle, EBV-positive GC (AGS-BX1, SNU-719) and NPC (HONE1-EBV, C666.1) cells were treated with combination of hit compound (**41**, **EF24**) and GCb+VPA or GCb+VPA alone for 48-96 h. Treatment with SAHA and SB were included in the analysis. After treatment, the cells were pelleted, washed once with PBS and RNA were extracted using Trizol reagent (Invitrogen Life Science). A real time quantitative RT-PCR was performed with experimental details as described previously[27,29]. EBV mRNA lytic gene expression was compared with those of untreated (DMSO) control and presented as fold increase. Data were determined from three independent experiments.

Results

We have reported that the CLVA regimen (combined use of GCb and VPA), a FDA approved nucleoside analog and HDAC inhibitor, can significantly reactivate the latent virus in NPC tumor cells, changing from its dormant non-replicating stage into the lytic phase both in vitro and in vivo and applied this strategy in a clinical Phase I

trial with promising clinical responses[5,6]. We hypothesized that curcuminoid may act as EBV lytic induction sensitizer and by administering curcuminoid and CLVA regimen simultaneously, a higher percentage of latently infected cells would enter into lytic phase. Here, these structurally diverse curcuminoids were explored for their reactivating effects on the EBV lytic cycle.

To this end, we carried out a dose dependent screen at selected concentrations spanning 10 nM to 10 uM in AGS-BX1 cells carrying recombinant EBV and measured the green fluorescent intensity of lytic cycle induction by FACS (data not shown). Initial screening in AGS-BX1 cells showed that nanomolar concentrations of curcuminoids containing thiopyranone and piperidinone linkers (**211**, **219**, **41**, **227**, **EF24**) provided weak lytic induction activities (supplementary Figure S1). Compound **41**, **227** and **EF24** demonstrated strong Zebra and EA-D reactivation at 1.25 μM compound concentration (Figure 2B).

Curcumin and **PGV0** were able to stimulate lytic activity at higher dose (10 μM) (Figure 2C). PGVs containing cyclopentanone linkers (**PGV1**, **PGV5**, **THPGV0**) showed stronger EBV Zebra and EA-D activation with 1.25 μM being the minimal dose for lytic reactivation in HONE1-EBV cells (Figure 2C). To confirm curcuminoid induced reactivation of EBV lytic cycle, we also examined the expression of Zebra (immediate-early) and EA-D (early) proteins in both GC and NPC cells containing native EBV genomes (SNU-719 and C666.1). The experimental scheme to investigate compound induced lytic induction in NPC and GC cells is presented in Figure 2A. This initial assessment led to the selection of three hit compounds, namely the thiopyranone dioxides **41**, **227** and the piperidinone **EF24**, for further evaluation.

Different lytic induction effect of curcuminoids in gastric and nasopharyngeal carcinoma cell line carrying recombinant EBV

Two recombinant cell lines AGS-BX1 and HONE1-EBV, generated by infecting AGS (GC) and HONE1 (NPC) cells with recombinant EBV genome are widely used to study induction of the EBV lytic cycle in vitro. We observed that incubation of AGS-BX1 cells with hit compounds at 1.25 μM caused a time-dependent increased EA-D activation up to 48 h then plateaued by 72 h (Figure 2D). Strong visible EA-D band produced by AGS-BX1 induced with **EF24** for 48 h revealed that **EF24** is more potent than **41** and **227** (Figure 2D). These data indicated that the lytic induction kinetics varied between curcuminoids, the best and the fastest of which was **EF24**. In agreement with a previous study[21], we found similar kinetics of expression of EBV lytic proteins in NPC cells containing recombinant EBV, HONE1-EBV (data not shown). These data show that 48 h induction time is sufficient for EBV lytic reactivation from latency in carcinoma cells carrying recombinant EBV genomes (Figure 2A).

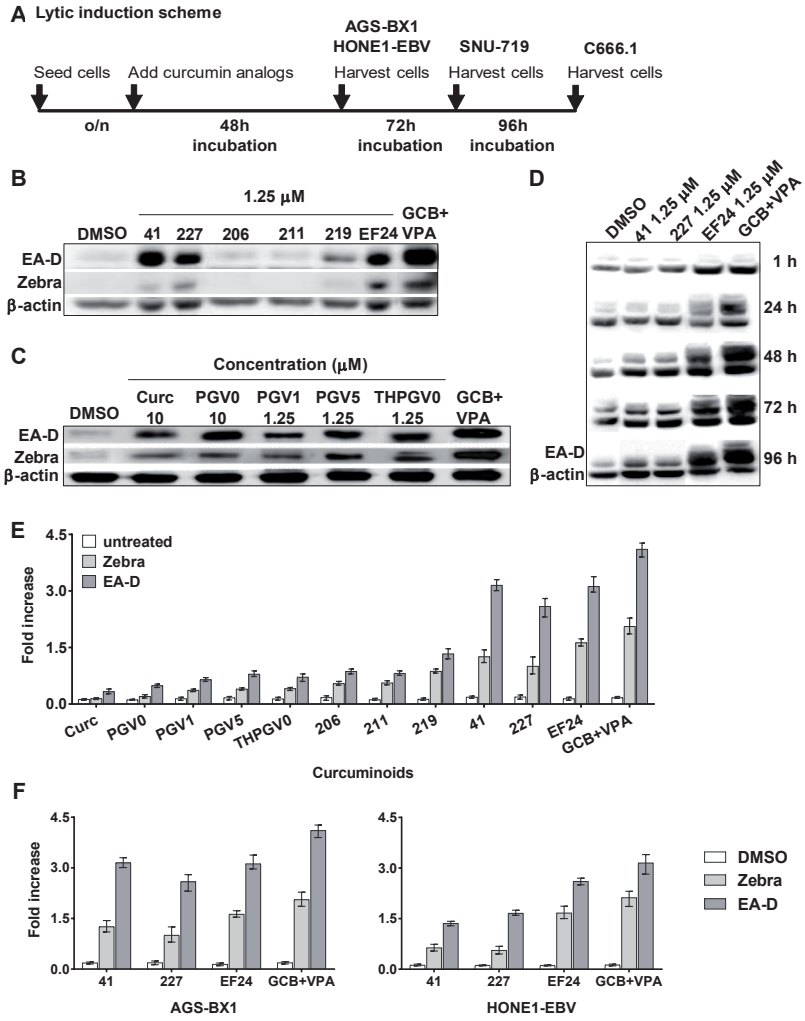

Figure 2. Different lytic induction effects of curcuminoids in gastric (GC) and nasopharyngeal (NPC) carcinoma cell lines transformed by recombinant EBV genomes. Curcumin and curcuminoids promote the accumulation of Zebra and EA-D lytic proteins in gastric (AGS-BX1) and nasopharyngeal (HONE1-EBV) carcinoma cells. A, Experimental scheme representing the curcuminoid treatment of EBV-positive GC and NPC cells. B, Treatment at 1.25 μM concentration resulted in strong lytic induction effects of hit compounds **41**, **227**, and **EF24**. C, Lytic induction effects of curcuminoids in HONE1-EBV cells. D, Expression of Zebra and EA-D lytic proteins in AGS-BX1 cells after treatment with 1.25 μM of hit compounds (**41**, **227**, **EF24**) for 1, 24, 48, 72, or 96 h or no treatment analysed by immunoblot. E, The relative level of EBV lytic proteins (Zebra, EA-D) induced by curcuminoids was assessed after normalisation with β-actin as loading control. Results are presented as fold increase of EBV lytic proteins of treated cells compared with untreated cells. F, The lytic induction effect of hit compounds (**41**, **227**, **EF24**) is more effective and intense in AGS-BX1 (evidenced by 1.5 to 3-fold increase) compared with that of HONE1-EBV (1.2 to 2-fold increase) cells. AGS-BX1 and HONE1-EBV cells treated with 3 mmol/L GCb and 0.3 mmol/L VPA were included as positive controls (B-F).

Figure 2E shows a quantitative blot scan analysis demonstrating that lytic induction varied among curcumin analogs with different linker modications. The immunoblot blot results also indicated that the CLVA regimen (GCb+VPA) provides more potent lytic induction than single dose curcuminoids alone (Figure 2E). We observed that at 1.25 μM concentration of each hit compound was sufficient to induce high expression of EBV immediate-early (Zebra) and early (EA-D) proteins in both AGS-BX1 and HONE1-EBV (Figure 2E). Incubation of the three hit compounds (**41**, **227**, **EF24**) in AGS-BX1 markedly induced the expression of Zebra and EA-D proteins at a level 1.5- to 2-fold higher than in HONE1-EBV (Figure 2E; left and right panels).

Similar results were found for EBV lytic reactivation visualized by fluorescence microscopy. Representative images of lytic induction by several curcuminoids in cell lines with recombinant EBV genomes are featured in Figure 3. In agreement with other studies[29-31], we found approximately 1–5% of untreated AGS-BX1 and HONE1-EBV cells show spontaneous lytic reactivation (Figure 3A, 3B). To analyze the percentage of cells entering the lytic phase, we counted the amount of cells expressing weak and strong green-fluorescence intensity and classified them into two categories; low and high EBV lytic reactivation (Figure 3B).

Among curcuminoids, **EF24** induced EBV reactivation at a comparable level to GCb+VPA's effect (62–69%) in AGS-BX1 cells, whereas curcumin itself only reactivated 10% of AGS-BX1 cells into the lytic cycle (Figure 3A). EBV lytic reactivation in HONE1-EBV prior to **EF24** and GCb+VPA treatment was demonstrated in Figure 3B. Approximately 57% of HONE1-EBV cells exhibited GFP-expressing EBV lytic reactivation upon lytic switch activated by **EF24** treatment. The lytic induction by GCb+VPA treatment was less in HONE1-EBV (45%, Figure 3C; right panel) as compared to AGS-BX1 cells (62%; Figure 3C; left panel). This indicates that cell background influences the permissiveness to induce EBV lytic reactivation. In addition, higher levels of spontaneous lytic reactivation of AGS-BX1[6,11,24,27] indicates that recombinant EBV in GC cells is more susceptible to chemical lytic inducing agents than recombinant EBV in NPC cells.

Curcuminoids better induce EBV reactivation in the carcinoma cells carrying recombinant EBV compared to natural EBV

Next, we tested whether hit compounds (**41**, **227**, **EF24**) could induce EBV lytic cycle in natural EBV genome-carrying GC (SNU-719) and NPC (C666.1) tumor cell lines. We included GCb+VPA[5], SAHA[17,19] and SB[18,19] as at optimal concentrations as positive controls. Lytic induction was achieved with all hit compounds in C666.1 with higher (2.5- to 3-fold increase) Zebra and EA-D activation (Figure 4A), compared to SNU-719 (Figure 4B). All three positive controls (SAHA, SB and GCb+VPA) induced strong EA-D activation

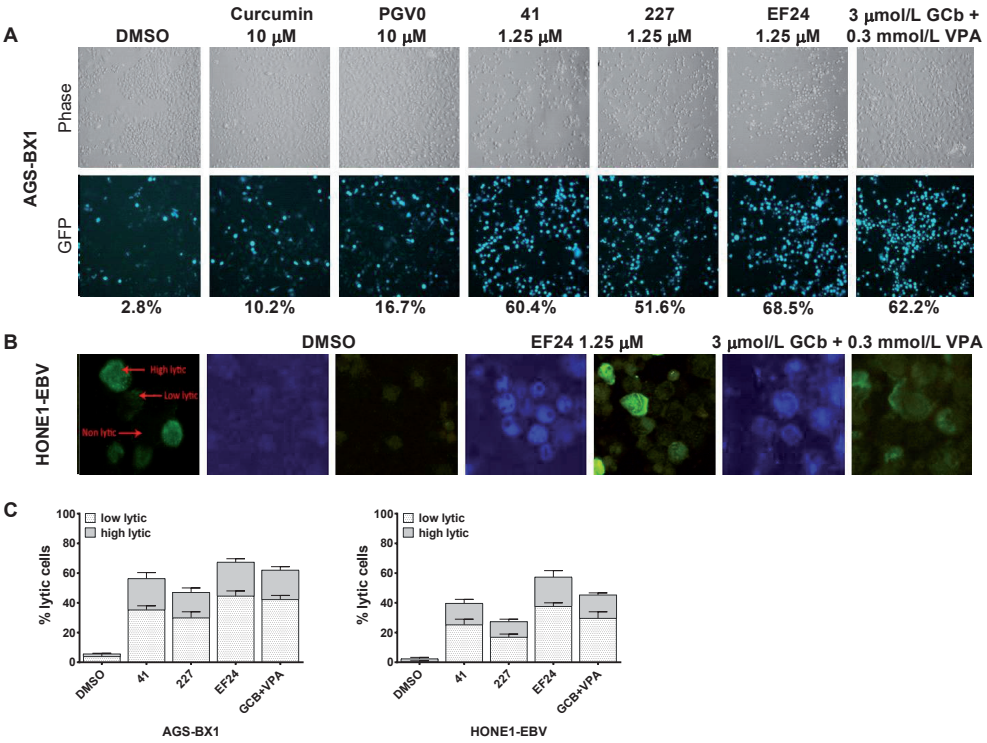

Figure 3. Percentage of AGS-BX1 and HONE1-EBV cells expressing EBV lytic reactivation following treatment with curcuminoids. AGS-BX1 cells were treated with several curcuminoids at their optimal lytic concentrations for 48 h. A, The cells expressing GFP-lytic EBV reactivation was visualized under a fluorescence microscope and the percentage of lytic cells was calculated. Approximately 3% of untreated AGS-BX1 cells expressed spontaneous lytic reactivation. B, Representative images of GFP-positive HONE1-EBV cells induced by compound **EF24** compared to positive control GCb+VPA. For quantification, GFP-expressing EBV lytic cycle was classified into two category: low lytic (weak GFP expression) and high lytic (strong GFP expression). C, The percentage of AGS-BX1 and HONE1-EBV cells induced into lytic cycle was estimated by calculating amount of cells expressing low and high lytic activation. Although most cells induced by hit compounds expressed low lytic, strong GFP expressions (high lytic activation) were observed in approximately 20–22% of AGS-BX1 and HONE1-EBV cells upon **EF24** treatment.

in both cell types, whereas only SAHA induced Zebra and EA-D protein expressions in SNU-719 (Figure 4B). Curcuminoids with cyclopentanone linkers (eg. **PGV0** 10 μM) induced EBV lytic activation in C666.1 NPC cells, but not in SNU-179 (data not shown).

Compound **41** and **227**, which induced strong EBV lytic cycle in AGS-BX1 and HONE1-EBV, did not show lytic induction effects in SNU-719 (Figure 4B). Unexpectedly, only **EF24** promoted weak EBV lytic reactivation in SNU-719 cells (Figure 4C). Overall these data indicate differential inducing effects for the individual compound when tested in different tumor cell backgrounds.

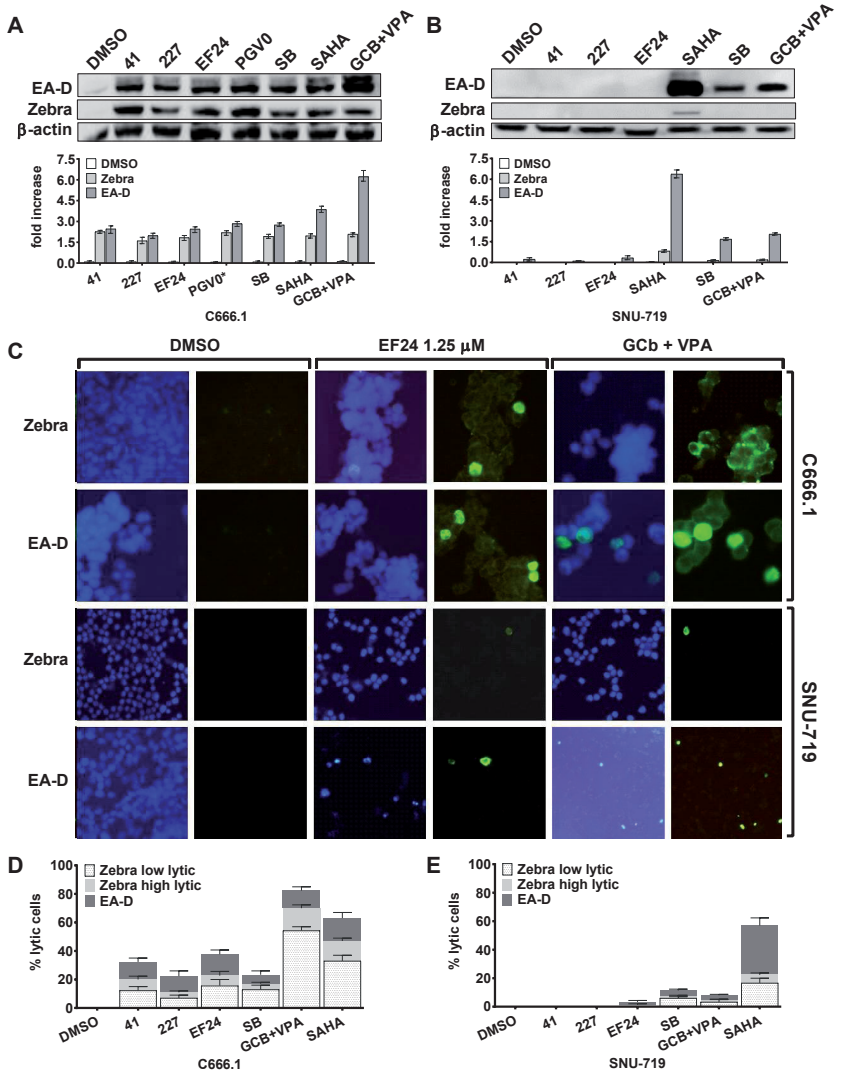

Figure 4. Curcuminoids have less lytic induction effects in natural EBV genome-carrying NPC and GC compare to recombinant carcinoma lines. Hit compounds at 1.25 μM lytic concentrations were able to induce EBV lytic cycle in natural EBV genome-carrying NPC (C666.1) but not GC (SNU-719) cells. The expression of Zebra and EA-D proteins upon treatment with curcuminoids analysed by immunoblot and immunofluorescence staining. Proteins extracted from cells cultured with 5 μM SAHA, 3 mM SB, or 3 mmol/L GCb and 0.3 mmol/L VPA were loaded as positive controls. Cellular β-actin served as loading control. The induction of lytic EBV by curcuminoids is more effective in C666.1 (A) than SNU-719 cells (B). SNU-719 expressed strong EA-D lytic proteins upon treatment with positive controls SAHA, SB and GCb+VPA. Representative immunofluorescence images of two EBV lytic cycle proteins in C666.1 and SNU-719 cells (C). Strong green fluorescence signals of Zebra and early antigen EA-D were identified upon **EF24** treatment in C666.1. Percentage of cells expressing Zebra and EA-D lytic proteins in C666.1 (D) and SNU-719 (E) cells. Zebra activation was detected in nearly 23% of C666.1 cells, whereas EA-D was expressed by approximately 14% of C666.1 cells upon EF24 treatment (D). Approximately 70% of C666.1 (D) and 5% of SNU-719 (E) cells expressed EBV lytic proteins upon GCb+VPA treatment.

We further analyzed the percentage of cells being induced into lytic cycle via immunofluorescent staining of C666.1 and SNU-719 cells on treatment with 1.25 μM concentration of hit compounds together with three positive controls. Representative immunofluorescence images of C666.1 and SNU-719 cells treated by **EF24** and GCb+VPA are shown in Figure 4C. As previously defined by Wildeman[6], the intensity of Zebra staining are categorized into low and high EBV-lytics, whereas the EA-D staining was defined as a positive or negative signal, being a bright nuclear staining in all reactivating cells. Consistent to immunoblot results, we only detected limited expression of EA-D-positive SNU-719 cells induced by **EF24** (Figure 4C). On the contrary, in NPC cells containing native EBV genomes **EF24** induced the expression of Zebra (range from 15% to 20%) and EA-D (range from 12% to 18%) activation (Figure 4C, 4D). SAHA demonstrated strong lytic induction effects in up to 63% of C666.1 (Figure 4D) and approximately 57% of SNU-719 cells (Figure 4E).

Interestingly, treatment with GCb+VPA combination strongly induced the expression of Zebra-positive C666.1 cells up to 82%, whereas only approximately 12% of C666.1 cells expressed strong EA-D activation (Figure 4C, 4E), which is consistent to previous studies[6,21]. In contrast to C666.1, the CLVA drugs only induced weak (8%) EBV lytic activation in SNU-719 (Figure 4C, 4E).

In summary, these data indicate that GC and NPC cells carrying recombinant EBV genomes particularly AGS-BX1 cells are more inducible for EBV reactivation by curcuminoids compared to GC and NPC cells with endogenous EBV. Furthermore, although **41** and **227** exhibit cell line-dependent induction, **EF24** could induce EBV lytic cycle in all the EBV-positive carcinomas tested at micromolar concentrations. Of the three hit compounds, **227** showed the least lytic induction effect, therefore for further analysis, we focused on **41** and **EF24**.

Analysis of cell viability at different concentrations of EBV lytic inducers

To examine direct cytotoxic effect of **41** and **EF24** in GC and NPC cells, we performed MTT assays at 48 hours post-treatment. Compound **41** and **EF24** demonstrated higher toxicity in GC (Figure 5A, 5C) and NPC (Figure 5B, 5D) cells containing recombinant EBV genomes (AGS-BX1 and HONE1-EBV) than the EBV-negative counterpart (AGS, HONE1, Figure 5A-D) at lytic induction concentration (1.25 μM), demonstrating EBV-specific effects of these curcuminoids.

Compared to SNU-719 cells (Figure 5A, 5C), C666.1 cells treated by **41** and **EF24** demonstrated high lytic induction with less toxicity (Figure 5B, 5D). Two strong lytic induction agents, SAHA and GCb+VPA demonstrated cytotoxicity effects in a dose-

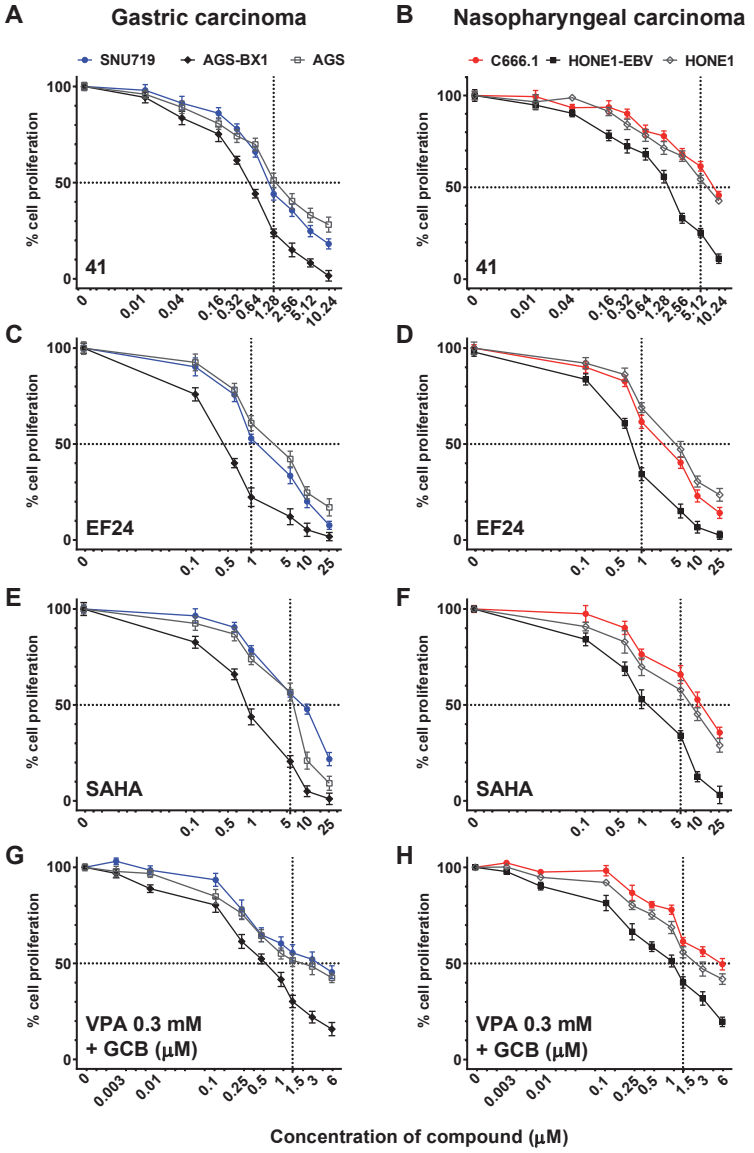

Figure 5. Naturally infected EBV positive carcinomas were more resistant to cytotoxicity effect of curcuminoids than those of recombinant EBV carcinoma cell lines. Cytotoxicity effect of hit compounds (**41**, **EF24**) was evaluated in GC (A, C) and NPC (B, D) cells. Treatments with SAHA (E, F) and GCb+VPA (G, H) were included as controls. AGS (EBV-negative), AGS-BX1 (recombinant EBV) and SNU-719 (native EBV) represent GC cells whereas HONE1 (EBV-negative), HONE1-EBV (recombinant EBV) and C666.1 (native EBV) represent NPC cells. All cells were incubated with various concentrations of lytic induction agents for 72h and cell proliferation of treated cells was determined by MTT assay. Results are expressed as percentages of treated cells compared with those of untreated cells and data from three independent experiments are presented. Standard deviation is shown in error bars. Dot line represents IC50 of compound/regimen in GC (A, C, E, G) and NPC (B, D, F, H) cells.

and cell line-dependent manner (Figure 5E-H). Histone deacetylase inhibitor (SAHA) displayed higher toxicity than GCb+VPA in GC cell lines (Figure 5E), but SAHA was less toxic in NPC cell lines (Figure 5F), whereas GCb+VPA demonstrated minimal toxicity in both GC (Figure 5G) and NPC (Figure 5H) cell types.

Among lytic induction agents tested in this study, we found that **EF24** (Figure 5C, 5D) exhibited greater toxicity than SAHA (Figure 5E, 5F) even though its lytic inducing activity was lower than that of SAHA (Figure 4). This is consistent with previous studies on antiproliferative activity of **EF24** against many type of cancer cells in vitro[17,20,30]. These findings suggest that the virus lytic inducing effect is not related to cytotoxic potency of the compound. EBV-associated carcinoma cell lines containing recombinant EBV genomes (AGS-BX1, HONE1-EBV) are more prone to killing (Figure 5A, 5C, 5E), whereas native EBV genome-carrying C666.1 did not show virally-mediated killing at lytic inducing concentrations (Figure 5B, 5D, 5F).

Taken together, we conclude that although EBV lytic cycle induced by curcuminoids is easily induced in cell lines artificially infected by EBV (AGS-BX1, HONE1-EBV), the authentic EBV-positive GC and NPC cell lines (SNU-719, C666.1) appear more resistant and may represent a more natural model to study cytolytic viral activation in vitro.

Synergistic effects of curcuminoids in combination with CLVA regimen

We previously reported that both GCb and VPA can induce the lytic cycle of EBV in NPC cell lines, and their combination resulted in a strong synergistic effect[5,6]. Therefore, our next aim was to investigate a possible cooperative lytic induction effect of curcuminoids (**41**, **EF24**) and GCb+VPA in GC and NPC cells. As **41** and **EF24** significantly inhibit cell proliferation and reduce cell viability, we wonder whether these hit compounds in combination with GCb+VPA can enhance the cell killing. GC and NPC cells were treated with 1.25 μM concentration of hit compounds (**41**, **EF24**) and/or combination GCb+VPA. Treatment with SAHA and SB were included as positive controls in each cell line and cell viability was measured by MTT assay for 96 h. At 48-96 h post-treatment, **41** and **EF24**synergistically enhanced the cell killing effect of GCb+VPA in GC (Figure 6A, 6B) and NPC (Figure 6C, 6D) cells.

Similar to cell killing effect of SAHA, these three compounds in combination (**41**, GCb+VPA; **EF24**, GCb+VPA) significantly reduce viable AGS-BX1 (Figure 6A) and HONE1-EBV (Figure 6C) cells within 48 h, SNU-719 cells within 72 h (Figure 6B), and C666.1 cells within 96 h (Figure 6D). The synergistic cytotoxic effects of **EF24** combined with GCb+VPA in C666.1 cells was higher than SAHA (Figure 6D). In contrast to other lytic induction agents, SB induced tumor cell death only in GC and NPC cells carrying recombinant

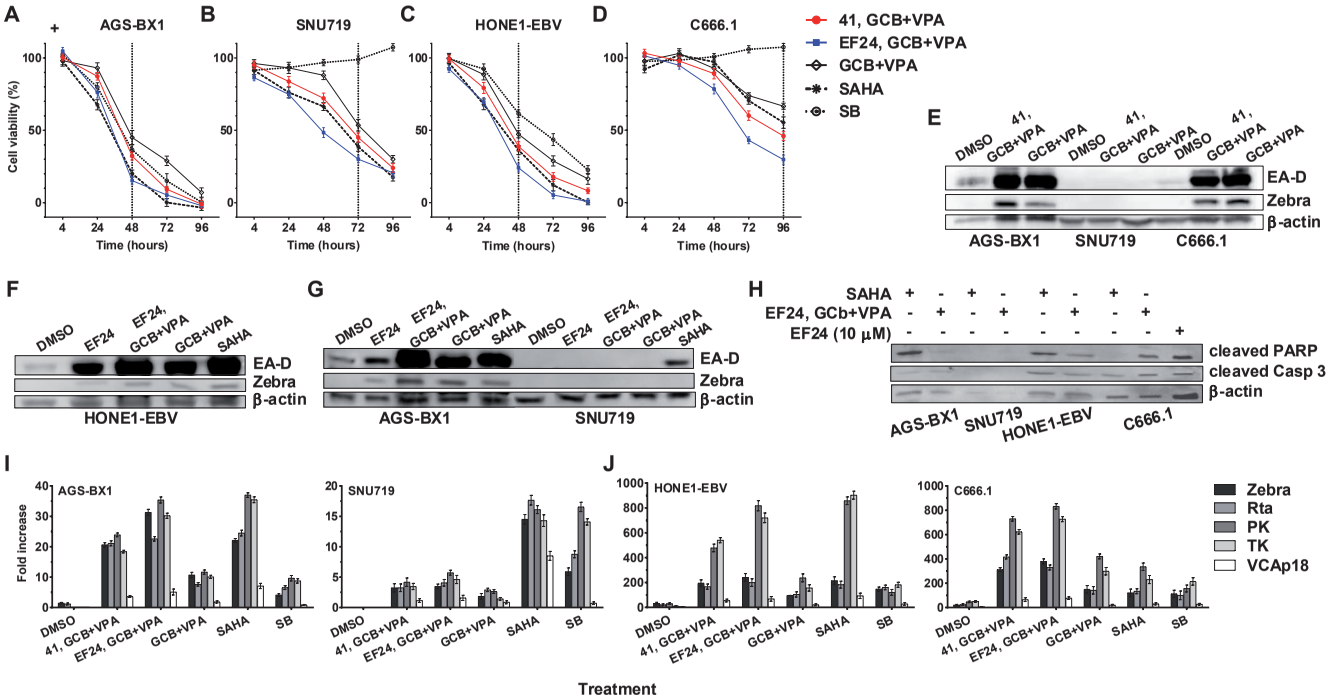

Figure 6. Combination of hit compounds and CLVA regimen synergistically induce EBV lytic cycle, significantly enhanced cell death and expressed apoptosis markers. Synergistic effects of hit compounds and CLVA regimen were measured by MTT assay, immunoblot and RT-qPCR quantification. EBV positive carcinomas: A, AGS-BX1; B, SNU-719; C, HONE1-EBV; and D, C666.1 cells were treated with hit compounds (**41**, **EF24**) in combination with GCb+VPA or GCb+VPA alone for 48-96 h followed by measurement of cell viability relative to untreated cells by MTT assay. Treatment with SAHA and SB were included as controls. Vertical dot line represents treatment time in each cell line. E, AGS-BX1, SNU-719 and C666.1 cells were treated with combination of **41** and GCb+VPA or GCb+VPA alone for 48-96 h and the expression of Zebra and EA-D lytic proteins were analysed by immunoblot. Cellular β-actin served as loading control. F, HONE1-EBV cells were treated with EF24 and GCb+VPA, GCb+VPA alone or SAHA. G, **EF24** significantly enhanced lytic induction capacity of CLVA regimen in EBV recombinant gastric carcinoma AGS-BX1, but not natural EBV genome-carrying SNU-719 cells. SAHA showed its capacity to induce EBV lytic in SNU-719. (G, right panel). H, EBV-positive AGS-BX1, SNU-719, HONE1-EBV and C666.1 cells were treated with 5 μM SAHA (lane 1, 3, 5, 7) or **EF24** and GCb+VPA in combination (lane 2, 4, 6, 8) for 72 h followed by detection of expression of cleaved PARP and cleaved caspase-3 by immunoblot analysis. AGS-BX1 cells treated by 10 μM **EF24** was used as a control (lane 9) and cellular β-actin served as loading control. RNA profiling of EBV-positive GC (I) and NPC (J) cell lines treated by either combination of hit compounds (**41**, **EF24**) with GCb+VPA or GCb+VPA alone. SAHA and SB treatments were used as positive controls whereas treatment with DMSO was used as negative control. RNA profiling of lytically induced-AGS-BX1 (I, left panel); -SNU-719 (I, right panel); -HONE1-EBV (J, left panel) and -C666.1 (J, right panel) cells showed increased levels of immediate early (Zebra and Rta) and early (PK, TK) lytic transcripts, whereas late lytic (VCA-p18) transcripts remained low or undetectable in all treated GC and NPC cells.

EBV genomes but not in carcinomas with native EBV, despite having capacity to induce EBV reactivation in these cells (Figure 4A, 4B). Our data indicate that curcuminoids can enhance cell death when combined with GCb+VPA in EBV-associated carcinomas.

To determine whether curcuminoids can induce lytic EBV and enhance the killing effect of CLVA regimen (GCb+VPA), the EBV lytic protein and lytic mRNA expression were examined by immunoblot and real time quantitative RT-PCR[6,27,29] in EBV-positive GC and NPC cells treated with **41** or **EF24** as adjuvant to CLVA treatment. We found that although **41** could cooperate with GCb+VPA, both demonstrated lytic reactivation on their own (Figure 2G, 4A, 4D), **41** did not enhance the activation of EBV lytic proteins (Figure 6E), whereas combining **EF24** with GCb+VPA not only caused enhanced cell death (Figure 6A-D) but also significantly enhanced the expression of EBV lytic proteins in both EBV-positive GC and NPC cells (Figure 6F, 6G, Supplementary Figure S2).

We also examined the effects of pre-treatment with curcuminoids prior to GCb+VPA on EBV lytic activation in the GC cell line AGS-BX1. Following pre-treatment for 24 h and a further 24 h GCb+VPA incubation, cell extracts were collected for detection of lytic proteins by immunoblot analysis. Surprisingly, the induction of EBV lytic proteins (Zebra and EA-D) in response to CLVA (GCb+VPA) treatment was downregulated by pre-treatment with curcuminoids for 24 h (Supplementary Figure S3). Curcuminoids, which activated EBV in C666.1 (Figure 6E) HONE1-EBV (Figure 6F), and AGS-BX1 (Figure 6E, Figure 6G) cells, synergized with CLVA regimen when used as adjuvant and added simultaneously. Taken together, our data suggest that the activation of EBV lytic cycle by curcuminoids is independent of their cytotoxic potency and simultaneous administration of curcuminoids with CLVA regimen provides the best lytic induction.

Combination of curcuminoid and CLVA effect on apoptosis and EBV lytic protein expression in gastric and nasopharyngeal carcinomas carrying artificial EBV genomes

The variation of curcuminoids in activating EBV lytic cycle in different cell types reveals possible mechanistic differences in cell line-dependent lytic reactivation. As replication of EBV can be triggered by apoptosis[31], we conducted experiments to investigate the effect of curcuminoids and SAHA on cell apoptosis. The expression levels of proteolytic cleavage of caspase-3 and PARP, both markers of cell apoptosis were analyzed by immunoblot. To accomplish this, EBV-positive GC and NPC cells were treated with combined **EF24** and CLVA regimen. SAHA treatment was included as additional lytic induction control[21,24]. Since the cytotoxic effect of many drugs is greater in AGS-BX1, we used AGS-BX1 treated with 10 μM **EF24** to identify increased levels of both apoptotic markers.

Effective induction of the EBV lytic cycle in HONE1-EBV (Figure 6F) and AGS-BX1 (Figure 6G; left panel) cells was confirmed by the expression of Zebra and EA-D lytic proteins following combined **EF24**, GCb+VPA or SAHA treatments. AGS-BX1 and HONE1-EBV cells treated with combined **EF24**, GCb+VPA did not show increased levels of both cleaved caspase-3 and PARP proteins. SAHA treatment showed a tendency to increase cleaved PARP protein levels in recombinant GC and NPC cell lines (Figure 6H). In contrast to AGS-BX1 cells, cleaved PARP protein levels were enhanced in C666.1 cells by combined **EF24**, GCb+VPA treatment but not by SAHA.

The relation between EBV lytic protein expression (Figure 6E) and apoptosis of EBV-positive cells (Figure 6H) was more clearly observed in C666.1 cells. Interestingly, SNU-719 cells which relatively sensitive to cytotoxic effect of combined **EF24**, GCb+VPA treatment (Figure 6B) but resistance to EBV lytic reactivation (Figure 6G; right panel) did not express either cleaved caspase-3 or PARP protein markers (Figure 6H). Taken together, our data indicate that apoptosis is not a prerequisite for EBV lytic activation and vise versa. In agreement with previous studies[21,31], we observed apoptosis-associated EBV lytic reactivation in GC and NPC cells transformed by recombinant rather than native EBV genomes. It is possible that the apoptotic pathways in naturally infected EBV cell lines may exhibit some important differences compared to EBV recombinant cell lines.

EBV immediate-early and early rather than late lytic gene expression was induced by combination curcuminoid and CLVA regimen treatment in EBV-positive carcinomas.

Previous studies indicated that the combination of GCb+VPA could increase EBV RNA levels encoding Zebra, protein kinase (PK), thymidine kinase (TK), and small capsid protein (VCA-p18) in AGS-BX1 and C666.1 cells[6,27]. Our data show that the combination of curcuminoids (**41**, **EF24**) with GCb+VPA resulted in a slightly increase in immediate early (ZEBRA) and early (EA-D) protein levels in both the EBV-positive GC and NPC cells (Figure 6E-G). To determine whether a combination of curcuminoid with GCb+VPA treatment activates EBV lytic genes, we performed qRT-PCR[24] from EBV-positive GC and NPC cells that had been exposed to a combination of CLVA regimen and **41** or **EF24**. SAHA and SB treatments were included as additional controls.

In AGS-BX1 cells, curcuminoid **EF24** together with GCb+VPA increased mRNA levels relative to DMSO of immediate-early (Zebra ~30-fold; Rta ~20-fold), early (PK ~35-fold; TK ~30-fold), and late lytic mRNAs (VCAp18 ~5-fold) 48 h after treatment, whereas a treatment with GCb+VPA alone increased the the EBV lytic gene expression approximately 10-fold (Figure 6I). DMSO alone had little effect in all cells (Figure 6I, 6J) except SNU-719. When EBV-positive NPC cells (HONE1-EBV, C666.1) treated with **41** or

EF24 for 48 h together with the CLVA regimen, EBV lytic gene activation was increased even higher. Zebra mRNA activation abundance was ~200- to 370-fold, Rta was ~200- to 410-fold, PK was ~480- to 830-fold, TK was ~540- to 720-fold, and VCAp18 was ~54- to 77-fold (Figure 6J).

A combination of **EF24** with GCb+VPA induce higher EBV mRNA levels than **41** with GCb+VPA in all cells except SNU-719 (Figure 6I). SAHA treatment induced similar EBV mRNA levels as EF24 with GCb+VPA. Our data indicate that using curcuminoid as adjuvant for CLVA treatment remarkably increased the EBV reactivation at the mRNA level above GCb+VPA alone (Figure 6I, 6J) and the lytic induction effect of this drug combination (EF24 with GCb+VPA) is similar to SAHA. Taken together, curcuminoid **EF24** has the most potent in vitro EBV inducing activity and could acts as adjuvant for CLVA therapy.

Discussion

EBV is consistently present in every tumor cell of EBV-positive malignancies in a state of latency characterized by very restricted gene expression[1,4]. Standard care attributes to recurrences and disease progression in a certain proportion of EBV-positive NPC and GC cases[2-5]. Strategies for targeting the virus have been explored, including the concept of reactivating latent viral genomes in combination with antiviral therapy[5-9]. Anti-herpes viral drugs that rely on phosphorylation by viral thymidine kinase for conversion of the prodrug to its active form, are not effective during latent infection[5,9]. Several drugs such as 5-azacitidine (5-Aza; 5,9), GCb+VPA (6,7) and SAHA[21,24] have been used in combination with antiviral agents in clinical studies, however, it remains unclear if the response seen was truly related to lytic induction and ganciclovir sensitization of tumor cells. Thus, discovering new and toxicity-limiting EBV lytic inducing agents to boost viral reactivation and selective elimination of EBV-carrying cells merits investigation.

The possibility of using a dietary constituent such as curcumin to induce lytic cycle in latently-infected EBV cells has remained largely unexplored[12,13]. Novel curcuminoids wih enhanced solubility, improved safety and/or minimal side effects[15-20] offer the option as alternative or supportive approaches to the current CLVA therapy, either as single agents or in combination with other lytic inducers. The present study was conceptualized to determine the effects of curcuminoids on the induction of EBV lytic cycle.

We demonstrated that hit compounds (**41**, **227**, **EF24**) could effectively induce the EBV lytic reactivation in vitro. Importantly, we showed that **41** and **EF24** synergistically induced the activation of EBV lytic cycle in GCb+VPA-treated EBV-positive GC and

NPC lines. As a result, tumor cell viability significantly decreased, in particular by the combination of GCb+VPA and the piperidinone **EF24**. Taken together, our data suggest that the activation of EBV lytic cycle by curcuminoids is independent of their cell killing effects. Therefore, the data presented here may provide a novel adjuvant approach for targeted treatment of EBV-associated gastric and nasopharyngeal carcinomas.

Most existing lytic inducing agents are frequently cell line specific or reactivate limited number of cells[9-11]. Here, we observed a remarkable increase in EBV lytic reactivation and concurrent reduction in cell proliferation leading to apoptosis in cells containing recombinant EBV genomes (AGS-BX1, HONE1-EBV) upon treatment with **41**, **227** and **EF24**. Our data also showed that these compounds triggered EBV into the lytic state in natural EBV genome-carrying NPC cell line (C666.1) and reduced the total number of proliferating EBV-positive cells. The effects were much less pronounced in the natural genome-carrying GC cell line SNU- 719.

However, **41** and **EF24** did have anti-proliferative activity in SNU-719 cells and mediated enhanced anti-proliferative activity of GCb+VPA against EBV-positive tumor cells. Furthermore, we also observed that the anti-tumor activity of these compounds was not EBV-specific[16-19,20,30]. Nonetheless, GC and NPC lines harbouring lytic EBV were more sensitive to killing by these novel compounds. Hence, curcuminoids may have lytic inducing capacity to trigger EBV reactivation from latency independent of their anti-proliferative activities.

The mechanisms that limit the level and speed of virus reactivation in different cell types or between cells in the same culture population are not well understood[10,11,32,33]. In agreement with others[11,21,24], our study showed that SAHA is a potent lytic inducing agents in all types of EBV-positive cells, including NPC and GC cell lines (C666.1, SNU-719) harbouring native EBV genome. The prototype EBVaGC line SNU-719 appears least sensitive to lytic induction by curcuminoids. Taken together, we conclude that host cell specific factors are contributing to the latency program in SNU-719 cells[34] to ensure that the EBV-infected cell remains in a latent state following curcuminoid treatment.

Recently, we showed that GCb+VPA can trigger EBV lytic replication in NPC patients[6,7]. Considering that each compound has lytic inducing[6] and anti-proliferative activity[6,16] in EBV-carrying tumor cells, we thus investigate the possible synergistic effects of combined the curcuminoid (**41**, **EF24**) with GCb+VPA. Consistently, we observed that a combination of **EF24** and GCb+VPA effectively enhanced the reactivation of EBV lytic cycle in EBV-associated carcinoma cell lines, with the exception of SNU-719 cells. Reactivation was accompanied by a reduction of tumor cell viability. The synergistic

induction of EBV lytic cycle combined with enhanced cell death in NPC and GC cells carrying recombinant EBV genomes, reflect the enhanced susceptibility of the recombinant lines to combined treatment with GCb+VPA and **EF24**.

It is well accepted that lytic activation in itself may render the infected cells more susceptible to immune recognition and killing due to the expression of viral lytic-switch proteins, in particular Zebra[4,8,9,31]. Interestingly, while the curcuminoids promoted EBV lytic induction by GCb+VPA in EBV-positive cells when added simultaneously, they also showed inhibitory activity against EBV reactivation when added separately prior to GCb+VPA treatment (supplementary Figure S3). These findings support what other investigators have proposed, that apart from their potential as viral lytic activators (35), natural dietary compounds such as resveratrol[36], sulfronane (SFN)[37] and luteolin[38] inhibit the EBV lytic cycle when added prior to viral reactivation by chemical treatment.

RNA profiling revealed that a combination of a curcuminoid (**41** or **EF24**) with GCb+VPA induced immediate-early and early lytic rather than late lytic gene expression. As demonstrated in this study and in agreement with previous publications, very low or undetectable levels of EBV late lytic gene expression upon EBV reactivation by chemical treatment suggestive of abortive lytic replication[21,27,29,39]. Taken together, we conclude that a simultaneous combination of a curcuminoid with CLVA regimen at acceptable doses may enhance EBV reactivation and provide an attractive therapeutic strategy for EBV-associated malignancies.

As such, we propose the type of cells based on their susceptibility to different types of stimuli (Supplementary Figure S4). Based on their susceptibility to chemical stimuli, EBV-positive lines can be divided into two types; permissive and less permissive cells. Strong lytic inducing agents such as HDAC inhibitor SAHA, EF24 and GCb+VPA can mediate the full lytic cascade in permissive cells such as GC and NPC lines harbouring recombinant EBV genomes (AGS-BX1, HONE1-EBV), whereas native EBV genome-carrying GC and NPC cells (SNU-719, C666.1) are less permissive to lytic stimuli. Thus even strong lytic inducers may not be able to reactive EBV in cells of this type.

Conclusions

In conclusion, our study identified two curcuminoids bearing the thiopyranone dioxide (**41**) and piperidinone (**EF24**) scaffolds that serve as putative EBV lytic activators in latently infected EBV-positive carcinomas. **EF24** has the potential to act as an adjuvant to enhance EBV reactivation induced by CLVA regimen (GCb+VPA) in EBV-associated

gastric and nasopharyngeal carcinomas.

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Supplementary

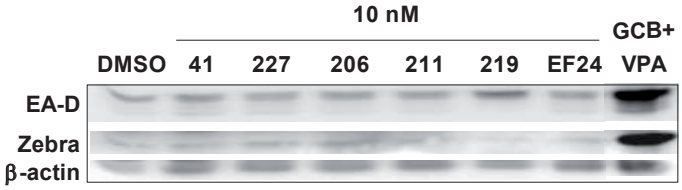


Figure S1. Curcuminoids induced weak EBV lytic reactivation at low (10 nM) concentrations.

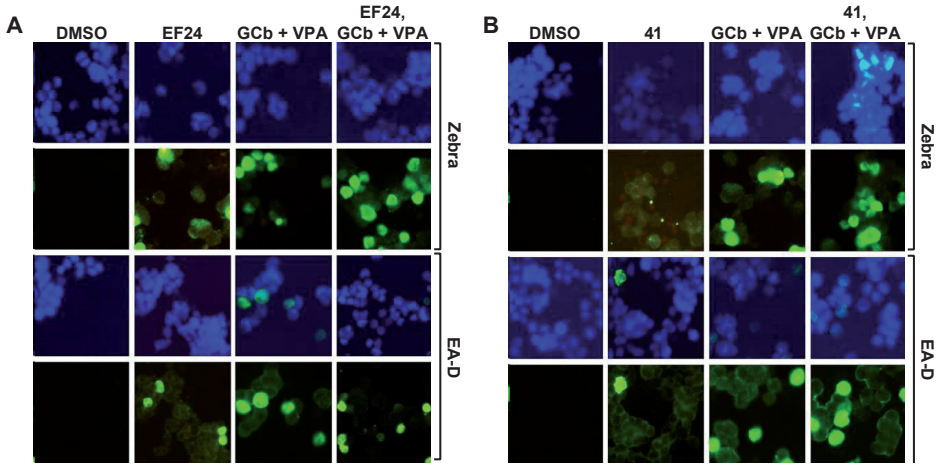


Figure S2. Representative immunofluorescence images of C666.1 cells treated by hit compounds (41, EF24) alone or in combination with GCb+VPA. A, C666.1 expressed Zebra and EA-D lytic proteins upon **EF24** treatment alone or in combination with GCb+VPA. B, C666.1 expressed Zebra and EA-D lytic proteins upon **41** treatment alone or in combination with GCb+VPA. Increased number of cells expressing Zebra and EA-D lytic proteins upon co-treatment with **EF24**, GCb+VPA (left panel) or with **41**, GCb+VPA (right panel) were shown.

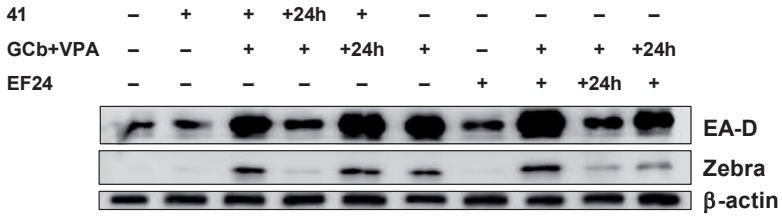
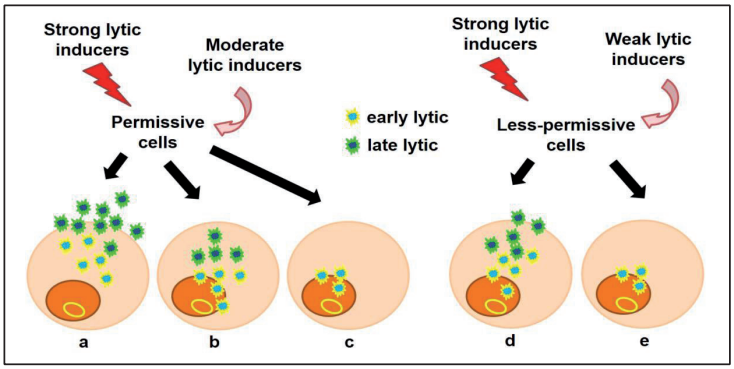


Figure S3. Pre-treatment with curcuminoid inhibits EBV lytic cycle induced by CLVA regimen. C666.1 cells were treated with hit compounds (**41**, **EF24**) 24 h before and after GCb+VPA administration. The cells were collected after 96 h followed by immunoblot detection of Zebra and EA-D proteins. Treatment with hit compounds or GCb+VPA alone for 96 h were included for comparison. When administered simultanously with CLVA regimen, hit compounds (**41**, **EF24**) sinergistically induced EBV reactivation. Curcuminoid antagonized the lytic induction effect of GCb+VPA when administered 24 h prior GCb+VPA treatment.



Strong lytic inducers : SAHA[(a,d)], EF24 and GCb+VPA[(a,d)]
Moderate lytic inducers : 41[(b)], EF24[(b)], 227[(b)], PGV0[(b)]
206[(c)], 211[(c)], 219[(c)],
PGV1[(c)], PGV5[(c)], THPGV0[(c)], curcumin[(c)]
Weak lytic inducers : 41[(e)], EF24[(e)], 227[(e)], PGV0[(e)]

Permissive cells : AGS/BX1, HONE1-EBV, C666.1
Less-permissive cells : SNU-719

Figure S4. Propose classification of EBV lytic induction in vitro. We classified lytic induction agents into three categories: strong, moderate, and weak lytic inducers. There are two types of EBV-positive lines: permissive cells consisting of carcinoma lines containing recombinant EBV genome eg. AGS-BX1 and HONE1-EBV and less-permissive cells such as natural EBV genome-carrying C666.1 and SNU-719 cells. Strong lytic induction agents can significantly induce full EBV lytic cycle both in permissive (A) and less permissive cells (D), whereas moderate lytic induction agents are only able to activate EBV lytic cycle in permissive cells (B, C). In contrast, weak lytic induction agents may be able to induce weak EBV lytic reactivation in permissive cells with low percentage of sporadic lytic protein expression, however weak lytic induction agents are unable to induce EBV lytic reactivation in less-permissive cells (E).

7

General discussion and future perspectives

Diagnostic findings using current EBV non-invasive biomarkers lack of correlation to tumor behavior

This thesis reports the use of EBV products for NPC management that includes not only disease diagnosis but also future targeted therapy. The main hindrance in combating NPC is the difficulty in early detection, accurate prognosis and treatment monitoring of the disease[1]. Prior studies done by us and others[2-4] concluded that although IgA- EBNA1 is a sensitive biomarker for identifying NPC (**Chapter 2**, **3**), false-positive results caused by past infections in endemic areas limit its clinical use for NPC diagnosis[3-6]. However, individuals that maintain these EBV antibodies at high levels for about 1–3 years may have early-stage NPC preceding the symptomatic clinical presentation[7]. This indicates the potential value of EBV-IgA serology for early identification of NPC (**Chapter 3**) during surveillance program, which is now being evaluated in large field screening studies in China[8].

Circulating cell-free and fragmented EBV-DNA was identified by some groups as promising diagnostic and prognostic marker for NPC[4,9-12]. However, we and others found that many patients with NPCs had low or undetectable levels at diagnosis (**Chapter 2**), even when presenting with bulky tumors, limiting the value of EBV-DNA load measurement in blood or plasma as screening marker[4,5]. Patients with persistently positive EBV-DNA levels in monthly repeated Q-PCR tests during survailance program are considered at risk of developing NPC and this marker may be used to confirm EBV-IgA serology-based NPC-risk screening[12,13].

Despite their potentials as biomarkers for screening individuals at risk of NPC in endemic regions, both current EBV serological (IgA-EBNA1) and molecular (EBV-DNA) biomarkers may not always represent tumor behavior and could theoretically detect asymptomatic infections[3,7-9,12].

During and following treatment for NPC, a significant (>30-fold) decrease in overall EBV-DNA level in whole blood can be considered as indicator of good response to therapy (**Chapter 2**). The prognostic value of circulating EBV-DNA load monitoring is confirmed in plasma, since NPC patients with high initial EBV-DNA levels tend to be less responsive to therapy and more prone to develop recurrent disease[11-14]. Differ from our findings in whole blood, in patients undergoing radiotherapy, an initial surge EBV-DNA concentration in plasma could be observed within the first two weeks of starting treatment, followed by a gradual decline[1,11,14,15]. Therefore, PCR-based cell-free plasma EBV-DNA detection may considered more specific than whole blood for monitoring treatment and for early detection of disease recurrence[10,13-15].

EBV microRNAs is secreted by living tumor cells and represent tumor behaviour: potential non-invasive biomarkers of NPC

Early detection of nasopharyngeal carcinoma targeting EBV gene products is critical for improving the 5-year overall survival rate. Existing methods including cell-free whole blood EBV-DNA and EBV IgA serology each have limitations (**Chapter 2**). Recent integrated analysis of differential cellular and EBV miRNA expression profiles in NPC cell lines, tissues and plasma samples highlight the potential of miRNA signature as novel biomarkers to improve the definition of symptoms for early detection of NPC[16-18]. Latest studies indicate the use of host and/or EBV-encoded miRNAs as important drivers in tumor-specific transcriptional regulation and as putative tumor marker, both in situ (brush or biopsy) and as secreted marker in the circulation (**Chapter 3**)[18-22]. However, it is as yet unknown whether these novel EBV biomarkers are effective for diagnosing early-stage NPC.

We first sequenced and profiled intra and extracelullar miRNAs in vitro with the purpose of identifying tumor specific EBV miRNAs in sera of patients with NPCs and confirmed their presence by nasopharyngeal brush samplings and tumor biopsies (**Chapter 3**). We compared the value of EBV miRNAs detection in different peripheral blood compartments (serum, plasma) and different specimens from patients with NPC (nasopharyngeal brushings and snap-frozen tumor biopsies) to predict for EBV-associated NPC. We and other investigators demonstrated that high levels of serum or plasma EBV miR-BART13 alone or in combination with miR-BART7 may serve as useful biomarkers for discriminating patients with NPCs from healthy subjects or patients with other diseases (**Chapter 3**)[23-26].

Some studies reported that detection of nasopharyngeal brush EBV miR-BART1 holds particular promise as diagnostic indicator of NPC and associates with advanced stages of the patients, thus making miR-BART1 a useful biomarker to predict NPC metastasis and to monitor tumor dynamics[22].

Hirai and coworkers reported that post-treatment detection of serum miR-BART17-5p is a potential biomarker of a poor prognosis[25]. Extracellular miRNA expression profiles are promising markers for precision diagnostics[27,28]. We identified EV-associated distribution of miR-BART13 in serum and plasma of patients with advanced NPC (**Chapter 3**). In contrast, Gourzones and colleagues observed that high concentration of plasma miR-BART17 does not co-purify with EVs[26]. Indeed, we believe that extracellular viral miRNAs merit further investigation for their diagnostic and prognostic potentials.

Analysis of cellular miRNAs (hsa-miRs) expressions in NPC tissues or plasma/sera may

improve diagnostic reliability of recurrent tumors as proposed by several groups although the results are variable and inconclusive[18,20,21,29]. Different from EBV miRNAs, we find that circulating hsa-miRNA biomarker signature are frequently confounded by lack of global consensus and are present in other malignancies, thus limiting the potential diagnostic and prognostic value of circulating cellular miRNA signature for NPC. Instead, in agreement with Zhang et al.[23] we found by multivariable logistic regression analysis that EBV 2-miR-BART signature (miR-BART7 and miR-BART13) has excellent diagnostic potential (**Chapter 3**). Further assessment of this approach is warranted in a larger cohort of patients, in particular patients at early-stage of disease.

Nasopharyngeal brush sampling as reliable and effective tool for detecting NPC-derived EBV

Due to NPC origin within the mucosal epithelia of the nasopharynx, we evaluated the diagnostic value of measuring EBV-DNA load in nasopharyngeal brush. It is proven that nasopharyngeal brush is an excellent marker for detection of NPC presence as confirmed by us and others in more recent independent studies (**Chapter 2, 3, and 4**) [15,30-32]. The results produced by us and other groups reveal that the degree of EBV-DNA positivity (number of EBV genome equivalents) and EBV gene expression (level and pattern of transcripts) was correlated with clinical status or treatment outcome regardless of the differences in sampling method, extraction protocol, genomic target of EBV-DNA fragment and normalization approach used[13,30,33].

Because the nasopharyngeal lymphoid tissue (Waldeyer's ring) is considered as the homing center for EBV carrying B-lymphocytes and is assumed to be a mucosal epithelial site of EBV virion production and secretion into saliva, it should be ruled-out that high EBV-DNA levels observed in nasopharyngeal brushings of NPC patients are derived from replicating mucosal virus rather than tumor cells carrying latent genomes (**Chapter 4**)[34].

The abundance of immediate-early RNA transcripts over late lytic transcripts in brushings and biopsies indicates that most reactivating events in the tumor-associated EBV genome are leading to interrupted or abortive infection, not producing new EBV genomes or virus particles as indicated by others[33,34]. Since evidence of full virion replication was nearly absent (i.e. barely detectable non-methylated EBV-DNA and late lytic transcripts) we concluded that most EBV-DNA in nasopharyngeal brushings are derived from tumor tissues and thus provides a reliable tumor marker, with evidence of limited abortive virus reactivation in the superficial layers of nasopharyngeal tissues (**Chapter 4**).

Early tumor detection using nasopharyngeal brush method is more powerful when skilled physician performs brush sampling to avoid/minimize bleeding during endoscopic examination[15,32]. However, due to limitations on CT scan or MRI imaging for differentiating T1 tumors from normal mucosa, early tumor development in the nasopharynx may be clinically occult, resulting sampling failure to reach the actual tumor[31,32]. This may lead to false negative or inconclusive results.

Another study reported that failure may be attributable to sampling inexperience and/or difficulty in controlling/limiting patient pain response or gagging when using the trans-oral brush approach[31]. Fortunately, in our study we noticed that the brush DNA load predicted tumor presence in the first sample obtained in parallel with the first biopsy although in some cases a repeated invasive biopsy was needed to obtain a pathological diagnosis **(Chapter 2)**.

Our study also demonstrates that separate nasopharyngeal brush taken via each nostril may increase positivity in small tumors, when no evidence of disease is shown by endoscopy[15]. A separate recent study indicates that in the same patient a nasal brush is less informative for EBV-DNA than the deeper nasopharyngeal brush when aiming at detecting (early-stage) NPC[31,32]. In sum, brush sampling is convenient to complement the serological tests and to confirm the presence of tumor in situ in patients with elevated circulating cell-free EBV-DNA levels.

Detection of multiple EBV biomarkers for NPC screening and diagnosis

A great effort was directed not only to the discovery and validation of new biomarkers aiming for more accurate detection but also to standardize the definition of early detection.[1,16,33,36.] Our studies confirmed that single EBV biomarker may not be effective for accurate early diagnosis of NPC (**Chapter 2, 3**).

As alternative to identifying aberrant serological responses to EBV in a risk population or to repeatedly detecting aberrant levels of fragmented cell-free plasma EBV-DNA as a single-marker non-invasive method suggested by others[4,8,9,12,37], we propose multiple EBV non-invasive biomarkers detection as screening and diagnostic tools. EBNA1 IgA detection followed by the detection of circulating cell-free EBV miR-BART13 may sensitively predict early NPC cases in endemic regions (**Chapter 3**). As more specific methods, PCR-based circulating cell-free EBV miR-BART13 alone or in combination with circulating cell-free miR-BART7 and EBV-DNA[24] can be applied a second line screening

and early NPC detection in individuals at risks when undetectable cell-free miR-BART13 or inconclusive IgA-EBNA1 occur (**Chapter 3**).

Next to non-invasive approach, nasopharyngeal brush sampling combined with EBV-DNA/RNA detection has gradually been established as a promising minimally-invasive method for confirmatory diagnosis of primary NPC[15,22,30]. Our studies indicated that EBV miRNA expression and EBV-DNA load in nasopharyngeal brush are a better indicator of NPC at primary tumor (**Chapter 2, 3 and 4**) compared to serology. This is not surprising given that serology provides indirect evidence of prior infection and largely is based on memory B-cell responses, whereas qPCR measures live tumor of active disease[34,36]. In addition, the diagnostic use of nasopharyngeal brush may be further increased by assessing a combination of molecular carcinoma markers in the same brush material, including viral load, tumor-specific EBV-RNA transcripts, EBV miRNA signature, host genomic methylation patterns, and other genetic abnormalities linked to NPC[38,39].

The detection of defined aberrations in methylated tumor suppressor gene (TSG) DNA in nasopharyngeal brush may also be used to further confirm the NPC tumor presence in situ[34,38,39]. In sum, all the above data and arguments indicate that nasopharyngeal brush sampling with EBV-DNA/RNA plus TSG-DNA methylation analysis has superior diagnostic and prognostic value over plasma or whole blood EBV-DNA load and IgA-EBNA1 or IgA-VCAp18 measurement[32,37-39,40].

The median interval between initial treatment and recurrence ranges from 1 month to 10 years; most patients in endemic region experience recurrence within 3 years of initial treatment[1,10]. As a follow-up monitoring marker for NPC patients with high pre-treatment EBV-DNA, plasma EBV-DNA is convenient, especially in those who have been subjected to different treatment protocols[10,11,13-15]. Stable elevated levels of nasopharyngeal brush EBV-DNA in patients with confirmed clinical recurrence or metastases within 6 months to 1 year after completion of radiotherapy underscore the potential of this method to provide stable and credible NPC marker test regardless disease stage[15]. Furthermore, the use of the nasopharyngeal brush EBV-DNA/RNA load post-treatment may limit its clinical value to detect recurrence due to difficulties to distinguish local residual tumor and post-radiation damage by endoscopy or imaging. In contrast, multiple EBV non-invasive biomarkers may overcome this issue in identifying disease relapse. Taken together, clinical potential of combined method detecting cell-free circulating EBV-DNA and EBV microRNAs as a non-invasive tool for real-time treatment monitoring and recurrent NPC diagnosis assessment warrants further investigation.

Further diagnostic considerations

The observational studies to evaluate the clinical utility of nasopharyngeal brush performed by us and other groups as discussed above, pose several complicating issues[15,31,32,41]. First of all, most studies were performed in a limited sample size with relatively small number of patients at early-stage (stage I/II tumors or T1/T2 subgroup) (**Chapter 2**). Second, we showed that circulating EV-enriched miR-BART13 is secreted by living tumor in patients with NPCs, however due to limited materials and sample size, we were not able to show the correlation of EV-enriched circulating miR-BART13 with EBV-DNA load in corresponding tumor biopsies to support our findings (**Chapter 3**). Thus, the correlation between multiple EBV biomarkers in serum and brush samples and the clinico-pathological characteristics of early-stage diseases requires further validation.

Unfortunately, most studies (including ours) did not present a diagnostic model for NPC using logistic regression that measure multi-EBV biomarkers in the same blood or brush materials (**Chapter 4**)[4,24,30,33,38,40]. Furthermore, limited information is available about the prediction value of blood or nasopharyngeal brush as a screening tool but also about the cost-effectiveness of a. the brush method b. repeated PCR tests in a wide population. This information would be helpful to more accurately guide the development of commercial tests to improve clinical intervention and treatment resulting in better prognosis of NPC.

Last but not least, most of studies represent patients from endemic populations. Cohort studies that compare NPC patients in different non-endemic regions are largely lacking[14,15]. Therefore, additional efforts should be undertaken to evaluate not only multiple EBV biomarkers but also simple and cheap method e.g. cytological examination in nasopharyngeal brush to provide information about early malignant events in the nasopharyngeal cavity[41].

In the future, additional well-designed clinical studies with larger groups of patients that cover endemic and non-endemic regions will be necessary to validate the diagnostic potential of multiple EBV biomarkers in serum and brush samples for early detection of primary or recurrent NPC[24,37,40] specifically in patients with undetectable or low EBV-DNA copies and equivocal serum EBV antibodies. In summary, multiple EBV-tests, including serology, circulating cell-free miRNA/DNA detection followed by nasopharyngeal brush sampling combined with DNA and/or miRNA analysis will attract most attention as the most predictive (diagnosis and prognosis) approaches in future.

NPC awareness and photodynamic therapy (PDT)

Although early-stage NPC is highly sensitive to radiotherapy, unfortunately, low-to-middle income countries face extremely limited access to this therapy, resulting in an unnecessary loss of lives[42,43]. With 70 percent of the world's cancer deaths expected to be in low-to-middle income countries by 2030, there is an urgent need to invest in early screening and combination therapy[44,45]. Due to the impact of national screening program and comprehensive treatment including intensity modulated radiation therapy (IMRT) performed in teaching hospitals in China, declines in NPC mortality have been observed in several endemic regions over the last two decades[1,8,10,32].

Whilst trends in NPC incidence have been reported as reasonably stable in high-risk areas of Southern China, many other economically developing countries in South-Eastern Asia still face a variety of difficulties in combating NPC[42-44,46]. Lack of awareness, lack of insurance, lack of treatment facilities and transportation problems contribute to this burden. Through education to develop awareness of NPC early-stage symptoms and to facilitate a national project in Indonesia for screening individuals at risks, downstaging of the tumor at presentation can be achieved[42,43,46].

Next to national NPC awareness program, the use of photodynamic therapy (PDT) for treating patients at early stage with nodal level 0 to 1 seems a realistic alternative to radiotherapy[46-48]. In contrast to high dose ionizing radiotherapy, PDT combines a photosensitizing agent with non-ionizing laser radiation (visible or near-infrared light) to produce cellular cytotoxicity in a small region of tumor. Importantly, PDT is affordable and requires small space, simple equipment and facilities[47].

In addition to tumor cell and tumor microenvironment effects that act to produce local tumor control, PDT can stimulate anti-tumor immune response that affects both local and systemic disease control[48]. In contract to radiotherapy, PDT would also save the patient from permanent tissue damage[47,48]. This strategy could reduce the treatment time from at least 6 weeks of daily-fractionated radiotherapy to a few days of PDT treatment[47].

PDT can be used as salvage treatment to reduce tumor volume and activity, prior to radiotherapy[48]. In countries like Indonesia this can be considered a major advantage. As most of patients are living in remote areas, they face difficulties to come to the hospital on a daily basis[42,43,46]. However, making PDT accessible in developing countries such as China and Indonesia is another big challenge. With an estimated 92 percent of new NPC cases occurring within economically developing countries in South-Eastern Asia[44],

adequate combinations of affordable (preferably) oral drugs with less toxicity that target viral functions in the tumor cells should be explored as an alternative treatment.

Standard and emerging treatment options for NPC

Although cure rates of NPC have been improved with the recent use of IMRT and neoadjuvant or adjuvant chemotherapy in top-level teaching hospitals, prognosis especially for patients with advanced loco-regional diseases in many hospitals in Southeast Asia remains discouraging[1,10,42,43,46]. Most of NPC patients with advanced diseases, regardless of initial responses to combined chemoradiotherapy, develop intolerable toxicity after receiving various modes of combined therapy which has led to debilitating long-term side effects[1,42,46]. In addition, their tumors acquire resistance to CRT[1]. Because of the lower toxicity and limited resistance, targeted therapy has increasingly been used for many common malignancies, although to date targeted therapy alone has not been approved as first line treatment for NPC[1,49,50].

There are ongoing trials evaluating the safety and efficacy of immune checkpoint inhibitor antibodies such as cytotoxic T lymphocyte associated antigen 4 (anti-CTLA-4 mAb) and programmed death-1 (anti-PD-1 mAb) on patients with head and neck squamous cell carcinomas (HNSCC) including NPC[10,49-52]. Although promising tumor outcomes have shown in treatment with anti-PD-1 mAbs, the prognostic and survival rates of patients with advanced NPCs after receiving anti-CTLA-4 mAbs remain inconsistent. Another approach specifically targets EBNA1 and LMP2 via CD8+ cytotoxic T-cells (CTL, so-called EBV-CTL) has shown promising overall responses in treating advanced metastatic or recurrent cancers including NPC[50,53].

In contrast, therapeutic monoclonal antibodies targeting epidermal growth factor receptor and vascular endothelial growth factor receptor (anti-EGFR and anti-VEGF mAbs) and small molecule tyrosine kinase inhibitors (EGFR-TKI and VEGF/VEGFR-TKI) for treating NPC have thus far proved disappointing. Clinical data have shown that these targeting therapies failed to improve overall and disease-free survival rates[51,52]. In sum, targeted therapy that include the use of therapeutic monoclonal antibodies and immunotherapies have not yet shown a sustainable anti-tumor response for NPC.

In addition to PD-1 or CTLA-4 expression levels, many host-related influences might account for the heterogeneous responses and failures during immunotherapies, such as nutritional status, smoking, and inflammation status[54,55]. Furthermore, mechanisms underlying EBV status in the immunosuppression process during monoclonal antibody

treatment is largely unknown[56]. In NPC, these tumor-associated antigens have co-evolved with the virus to evade immune recognition through various mechanisms, including CTL epitope mutations and impairing the major histocompatibility complex (HLA) pathways[50,53-56]. Hence, EBV-CTL remains "personalized" medicine that have to be matched with the patient's HLA-type[52,56].

From business standpoint, it is still not yet feasible to manufacture EBV-CTL in an economical fashion, certainly for use in developing countries. Taken together, therapeutic monoclonal antibodies targeting specific tumor-associated antigens and immunotherapy may unlikely to be suitable for NPC management in endemic regions.

EBV-targeted therapy in NPC

The constant presence of the viral genome in EBV-associated carcinomas suggests the feasibility of developing novel EBV-targeted therapies, so called cytolytic virus-activation therapy (CLVA)[51,52,57,58]. Our initial findings in a young dutch EBV-positive NPC patient with advanced recurrent disease concluded that a combination of a chemotherapeutic agent (5-FU) and valproic acid (VPA) was shown to induce viral lytic DNA replication, which was subsequently repressed by the anti-herpesvirus ganciclovir (GCV) treatment[58]. Therefore, for CLVA development purpose, we developed a multi-primed RNA detection method (**Chapter 5**) to evaluate EBV gene expression after lytic induction.

Due to improved efficacious therapeutics (e.g. drug that has lower toxicity on its optimal dosage, duration, and long-term effect) is needed, significant progress has been made in the research of CVLA by using both in vitro drug screening and animal experimentation[59-67].

By simultaneously assessing different EBV latency programs (**Chapter 5**) and quantifying EBV lytic transcripts before and after induction we are able to screen lytic induction agents in EBV-positive lymphoma and carcinoma cell lines (**Chapter 6**) using an optimized and validated multi-primed quantitative RT-PCR system[57]. This multi-primed quantitative RT-PCR system is also suitable for monitoring the dynamic of EBV gene expression during lytic induction therapy in animal model and patients[66-69].

In agreement with others[60,64,65,70], results in EBV-positive cell lines, animal model and patients[66-69] revealed that the latency mRNAs are expressed at the same level, whether the lytic cycle inducer, Zebra protein, is expressed or not[70] (**Chapter 5, 6**). Furthermore,

high viral load and detectable carcinoma marker BARF1 mRNA, tumor-derived latent viral mRNAs prevailed significantly over the (early and late) lytic transcripts.

Beneficial clinical responses of initial CLVA study in three Dutch patients with untreatable end-stage NPCs[68] have led us to continued a phase I/II clinical trial involving eleven patients, of whom were refractory to conventional therapy[69]. Clinical data showed that significant reduced tumor growth and improved overall survival rates were observed in some. In addition, these patients had far fewer side effects compared to standard treatments[68,69]. Currently, a multicenter phase I/II trial of SAHA and azacytidine (5-AZA) for locally recurrent NPC is ongoing (NCT 00336063). In sum, alternative epigenetic modulating agents combined with antiviral approaches becomes new perspective for breaking EBV latency .

Interestingly, CLVA oral regimen (GCb, VPA and valGCV) are now off-patent. Thus, this new approach may provide clinical and economic benefits for patients, especially in developing countries where chemoradiation treatment is very costly and access to advanced radiotherapy facilities is limited. Despite the limited use of CLVA, we hope that more extensive basic research, animal studies, and feasibility studies will be completed in next few years. Hence, CLVA efficacy for patients with NPCs or other EBV-associated cancers (e.g. EBV-positive BL and EBVaGC) can be evaluated in larger cohort and for a longer period of time.

Despite offering a new modality for cancer treatment, viral oncolytic therapy has several limitations. Most existing CLVA methods activate the lytic life-cycle in only a small percentage of latently infected cells, are toxic and are cell-type restricted[52,53,57,61]. Out of many known chemical activators of the EBV lytic cycle[57] only histone deacytylase inhibitors (HDACis) derived from butyrate analogues have been tested in several clinical trials[52,61]. However, the mechanism of killing as discussed elsewhere remains unclear[52,53,57,61-63].

Throughout the years, multiple agents have been described to trigger EBV lytic cycle, such as radiation and different chemotherapeutical agents - alone or in combination with HDACi[52,57]. Recently, Hui and colleagues have demonstrated that suberoylanilide hydroxamic acid (SAHA)[59] and romidepsin[60], which are alternative the HDACi, triggered EBV lytic cycle activation, led to apoptosis and tumor growth suppression of NPC thereby providing further experimental evidence for virus-targeted therapy against EBV-positive cancers. Research on this area is ongoing.

When developing or repurposing compounds for use in the systemic disease such as

cancer, it is important that they are relatively nontoxic with good (oral) bioavailability and preferably inexpensive. In recent years, small molecules and analogs of various natural compounds including curcumin demonstrated their potentials as activator of the virus lytic phase, which may facilitate the development of such compounds for use in CLVA therapy[61,63,64]. Our study revealed that the ability of curcumin and related compounds (curcuminoids) to reactivate latent EBV and to synergistically work with the standard CLVA regimen, provides new options for future clinical strategies (**Chapter 6**). In summary, the next few years will likely be exciting with the completion of in vivo studies of new compounds for EBV-targeted therapy.

Genetic diversity of EBV genome and its implications

The assessment of multiple EBV biomarkers which provides information-rich diagnostics for NPC will enable physicians to further develop early-onset individualized treatment plans for their patients[71,72]. In the era of comprehensive genomic profiling, novel specific EBV-targeted treatment in NPC using technologies such as immune therapy, immune modulation treatment and genetic or epigenetic therapy now becomes available for evaluation and well predict our ability to effectively target the clinically challenging aspects of locally recurrent and metastatic disease[72-74]. Alongside these therapies, the advent of personalized medicine raises the possibility of using molecular classification to subdivide NPC and thereby improve patient management and outcomes[73,75].

We[76,77] and others[78,79] have identified several key genomic alterations that link to NPC development and progression via a variety of mechanisms[16,34]. It is generally agreed that only few host genetic alterations are present in NPC compared to other epithelial cancers and the important role of EBV infection in altering cellular pathways to promote growth, metastasis and survival of infected NPC cells[34,72,74,80].

The number of infection-linked cancer deaths in the developed world is low, but one in five cancer deaths in developing countries are caused by infection[44,81,82]. Experts estimate that EBV vaccine could prevent two percent of all cancer cases worldwide[82-84]. Active research is ongoing in several laboratories to identify the best approaches. Understanding the pattern of EBV sequence variation is also important for EBV vaccine development [83,84].

Sequence variation that features the prevalent EBV strains across different populations including polymorphisms that can affect viral epitope presentation is identified by so-called next generation sequencing and polymorphism arrays. These sensitive and

reliable techniques may elucidate the relationship between EBV infection and the host cell pathobiology, such as regulation of viral gene expression program in normal vs. tumor cell types, cellular pathways modulated by viral proteins and cellular interacting partners for viral proteins.[16,34,79,80,90-95]

New high-throughput sequencing technologies are beginning to shed light on the contribution of EBV strain variation to the development of virus-associated NPC[73]. Focusing on the EBV genome regions, we identified high degree of sequence variation within the intronic regions of BART genes in isolates from Southern China and Malaysia[76,77]. This raises the intriguing possibility that BART miRNAs contribute to the development of NPC[96,97].

Secreted miRNAs, whether packed in EVs or not, might influence the behavior of stromal and immune cells, thus contributing to metastasis and immune escape[16,27,34,96]. It is therefore likely that variation in EBV oncogenes and BART genes found in Chinese descendants can contribute to the risk of developing virus-associated NPC[16,34,97]. Taken together, genome-wide analysis of EBV variation in individual patient with NPC may accelerate the development of future vaccine candidates as well as antivirals.

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Summary

When detected early, the survival rate for early-stage NPC is more than 80 percent. In Indonesia and other endemic countries in Asia, NPC tends to persist and re-occurs due to lack of NPC awareness and limited treatment options. Most patients with NPCs actually present with symptoms that are easily mistaken for innocuous problems. In Asian societies, many patients attribute these symptoms to being 'food-related disease' and often self-medicate with traditional medicine, thus delaying presentation to professional health-care facilities.

Known causes of NPC include genetics, regular intake of salted/dried fish and EBV infection. Infection with EBV is very common, affecting all human populations and is largely asymptomatic and life-long. It is unclear why this common virus triggers cancer in some people. Dietary exposure to nitrite and nitrosamines during childhood that trigger continuous virus reactivation in long-term period may play a role in the development of NPC. Different ethnic populations have NPC with incidence variations within endemic countries. Interestingly, the distribution of NPC around the world is changing, with Western countries forecast to get more cases. This trend is due to international migration of Chinese and their descendants to Europe and United States.

Most of our current knowledge on NPC has been generated in China and Western populations. As the socio-economic profile, life style and culture varies between each Asian country and are substantially different from western population, diagnostic and prognostic findings both in blood and tumor tissues, including NPC prediction rules, are increasingly shown to be 'setting specific'. Therefore EBV-associated NPC biomarkers must be validated in multiple populations before implementing them in clinical care in Asia.

Screening for NPC may improve early detection and outcomes. China and Taiwan have initiated population-based NPC screening programs whereas Indonesia still focuses on improving education program and national awareness. Effectiveness of EBV antibody screening among individuals at risk is sub-optimal and thus a combination with tumor biomarkers such as EBV-DNA and microRNAs may provide solution. These multiple tumor-related EBV biomarkers must be defined prudently, with appropriate customization for each region.

Despite screening programs in some countries, there is misunderstanding in parts of endemic and non-endemic regions about how NPC develops and can be treated, in

addition to a lack of awareness about the risks of traditional remedies. Geographical isolation, inadequate diagnostic and treatment facilities, and competing healthcare needs represent a significant problem to NPC prevention and control.

As researchers continue to identify and characterize biomarkers which predict disease presence and outcome, clinicians will be better poised to offer individualized effective care of patients' diseases. Oral drug formulation that both target virus gene products and enhance immune responses to cancer are becoming available and merits further clinical trials. NPC management in high-income vs. low-income countries should not affect ultimate efforts of reducing NPC mortality.

While NPC research is warranted in all domains of medical aspects, it is felt that for NPC patients in both endemic and non-endemic regions, needs are highest for diagnostic and prognostic studies. International clinical trials meanwhile need to include NPC patients not only from various Asian settings but also from population in Western countries to provide an insight into the effectiveness of new treatment modalities worldwide.

Nederlandse samenvatting

Bij vroege detectie is de overlevingskans voor NPC in een vroeg stadium meer dan 80 procent. In Indonesië en andere endemische landen in Azië heeft NPC de neiging door te groeien vanwege een gebrek aan bewustzijn voor NPC en beperkte behandelingsmogelijkheden. De meeste patiënten met NPC presenteren zich met symptomen die gemakkelijk worden aangezien voor onschuldige problemen. In de Aziatische samenleving schrijven veel patiënten deze symptomen toe aan 'voedsel-gerelateerde ziekten' en passen vaak zelfmedicatie toe met traditionele geneesmiddelen, waardoor een vertraagde gang naar en presentatie bij professionele zorginstellingen plaatsvindt.

Bekende oorzaken van NPC zijn erfelijke factoren, regelmatige inname van gezouten/gedroogde vis en EBV-infectie. Infectie met EBV treft alle menselijke populaties en is grotendeels asymptomatisch en levenslang. Het is onduidelijk waarom dit veel voorkomende virus kanker bij sommige mensen veroorzaakt. Langdurige blootstelling vanaf jonge leeftijd aan voedsel met nitriten en nitrosamines die latent virus reactiveren, kunnen een rol spelen bij het ontstaan van NPC. Verschillende etnische bevolkingsgroepen kennen NPC met grote variaties binnen endemische landen. Interessant is dat de verspreiding van NPC over de hele wereld aan het veranderen is, met de westerse voorspelling om meer gevallen te krijgen. Deze trend is te wijten aan de internationale economische migratie van Chinezen en hun nakomelingen naar Europa en de Verenigde Staten.

Het grootste deel van onze huidige kennis over NPC is gegenereerd in China en Westerse populaties. Omdat het sociaal-economisch profiel, de levensstijl en de cultuur binnen Azië verschillen van land tot land en ook aanzienlijk verschillen van de westerse populatie, kunnen diagnostische en prognostische bevindingen in zowel bloed- als tumorweefsel, waaronder NPC-voorspellingsregels, verschillen per regio. Daarom moeten EBV-geassocieerde NPC-biomarkers gevalideerd worden in andere populaties voordat ze geïmplementeerd worden in de klinische zorg.

Screening op NPC kan vroege detectie en uitkomst van behandeling verbeteren. China en Taiwan hebben op de bevolking gebaseerde NPC-screeningprogramma's geïnitieerd, terwijl Indonesië nog steeds gericht is op het verbeteren van het onderwijsprogramma en nationaal bewustzijn. De effectiviteit van screening op EBV-antilichamen bij risicopersonen is niet optimaal en daarom kan een combinatie met tumor-biomarkers zoals EBV-DNA of microRNA's een oplossing bieden. Deze tumor-gerelateerde EBV biomarkers moeten worden onderzocht met specifieke aanpassingen voor elke regio.

Ondanks screeningprogramma's in sommige landen, is er misverstand in delen van endemische en niet-endemische regio's over hoe NPC zich ontwikkelt en kan worden behandeld, naast een gebrek aan bewustzijn over de risico's en een beroep op traditionele remedies. Geografische isolatie, ontoereikende diagnostische apparatuur en behandelingsfaciliteiten en concurrerende gezondheidszorgbehoeften vormen een aanzienlijk probleem voor de preventie en bestrijding van NPC.

Dankzij het feit dat onderzoekers biomarkers blijven identificeren en karakteriseren die kunnen fungeren als surrogaten voor ziekteresultaten, zullen clinici beter in staat zijn geïndividualiseerde effectieve zorg aan te bieden. Orale geneesmiddelenformulering die virale genproducten als doelwit hebben en immuunrespons tegen kanker verbeteren zullen worden ontwikkeld en verdienen verdere klinische proeven. Het NPC-management in landen met hoge inkomens versus lage inkomens zou niet van invloed moeten zijn op de uiteindelijke inspanningen om de mortaliteit door NPC te verminderen.

Hoewel NPC-onderzoek gerechtvaardigd is in alle domeinen van medisch onderzoek, is men van mening dat voor patiënten in zowel endemische als niet-endemische regio's de behoeften het hoogst zijn voor diagnostische en prognostische studies. In internationale klinische studies moeten intussen NPC-patiënten niet alleen uit verschillende Aziatische omgevingen maar ook uit de bevolking in westerse landen worden opgenomen om inzicht te krijgen in de effectiviteit van nieuwe behandelingsmethoden wereldwijd.

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Curriculum vitae

Octavia was born in 1979, in Pancur Batu, North Sumatera, Indonesia. In 2003, she obtained her Bachelor of Science degree from School of Life Science and Technology of Bandung Institute of Technology (SITH-ITB) in Bandung, Indonesia. She started her study at Faculty of Medicine, University of Indonesia (FKUI) in Jakarta, Indonesia and obtained Master of Science degree in Biomedical Science in 2007. From 2007, she was appointed at Institute of Human Virology and Cancer Biology, University of Indonesia (IHVCB-UI) in Jakarta, Indonesia where she worked on bird flu project in collaboration with WHO. In 2008, she started her first traineeship at EBV group, Department of Pathology, VU medical center (VUmc), Amsterdam, the Netherlands and had the second one in 2010 where she learned about molecular diagnostic tests in nasopharyngeal carcinoma and other EBV-associated diseases. From 2008 until 2011, she worked at Cipto hospital as Clinical research associate, at SITH-ITB as Scientist, and at Hasan Sadikin hospital as Clinical research coordinator before starting PhD trajectory.

In 2011, she enrolled PhD program in the field of Oncology (Oncology Graduate School, VUmc) under the supervision of Prof. Dr. Jaap Middeldorp and Dr. D. Michiel Pegtel. Dutch cancer society (KWF) funded her research and she conducted the experiments in Cancer Center Amsterdam (CCA-VUmc) She received EMBO short fellowship grant and VUmc travel grant for postgraduate traineeship on ultrasensitive DNA sequencing strategies and liquid biopsy project at Anders Ståhlberg group at Sahlgrenska Cancer Center, Göteborg, Sweden. During her PhD training, she presenting her work in several international conferences in Europe, Asia, and United States. She made up the leap from academia to the corporate world. Currently, she works as the Clinical scientist at Microsure, a startup in the surgical robot technology which is based in Eindhoven, the Netherlands.

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“Nothing can dim the light which shines from within”

Maya Angelou