Epstein-Barr virus-associated malignancies

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CHAPTER 9

Summary, discussion and future perspectives

1. Summary and discussion

EBV plays an important role in EBV-associated malignancies in two distinct ways. First, it contributes to transformation of normal cells into cancer cells. Second, EBV-derived molecules can be utilized as biomarkers for diagnosis and prognosis. In this thesis, the pathogenetic mechanisms of multiple EBV-associated malignancies, i.e., Hodgkin lymphoma (HL), post-transplant lymphoproliferative disorders (PTLD) and nasopharyngeal carcinoma (NPC) were studied by focusing on the interaction between tumor cells and the tumor microenvironment and the value of different EBV-derived molecules as biomarkers.

The interaction between tumor cells and the surrounding environment has its history dated back to 1889, when Stephen Paget proposed the 'seed and soil' hypothesis. He described that tumor growth in secondary sites is not by chance. Metastasis to an organ can only take place when the tumor cells (seeds) encounter a conducive environment (soil) and colonize there. This is analogous to how plants spread their seeds to random directions, but seeds only grow if they fall onto the right soil. Although the 'seed and soil' theory was first described to show the metastatic preference of tumor cells, recent studies have shown that the microenvironment plays a similar if not more important role in the growth of tumor cells at the primary site. In **Chapter 2**, we reviewed current knowledge on the tumor microenvironment (TME) in three widely studied EBV-associated malignancies, i.e., HL, NPC and gastric carcinoma (GC). The composition of the TME varies greatly among different types of cancers. Of these, HL and GC are associated with EBV only in a proportion of the cases. Despite involvement of EBV as a common pathogenetic feature, these three malignancies all show a diverse infiltration of immune cells, often with many T cells. In the TME of EBV+ HL, the ratio of non-tumor cells to tumor cells is often larger than 100:1. Another distinct feature of the HL TME is the presence of rosetting T cells consisting mainly of T helper 2 (Th2) and regulatory T cells around the tumor cells. CD8+ T cells are more commonly found in the TME of NPC and EBV+ GC, however not all CD8+ T cells in the TME have cytotoxic properties. Nevertheless, it has become clear that T cells are an important component of the TME in all three EBV-associated malignancies.

The TME composition of these EBV-associated malignancies indicate that T cells in the TME do not induce effective anti-tumor responses but, together with other components of the TME including other immune cells and non-immune cells (e.g., fibroblasts and endothelial cells) provide support to the survival of tumor cells. This suggests that tumor cells have effective immune escape mechanism in play. Currently known immune escape mechanisms include, among others, at the level of antigen presentation by human leukocyte antigen (HLA) and modulation of antigen-dependent activation of T cells. Loss of HLA expression is one of the mechanisms by which tumor cells can avoid recognition by T cells.

Susceptibility to EBV-associated malignancies relies amongst others on genetic factors[1–6]. A main role has been shown for single nucleotide polymorphisms (SNPs) in the HLA genes. Such polymorphisms could result in marked differences in the efficiency to present specific antigens, including tumor cell-derived or EBV-specific antigens, and the efficiency of host immune response against EBV or tumor cells. Failure of the host immune system to effectively suppress EBV infection leads to a higher chance of malignant transformation of EBV-infected cells. In two independent studies on Mexican-American and French families, higher serum/plasma anti-EBNA-1 antibody levels were associated with SNPs within the HLA class II regions[7,8]. Multiple HLA alleles were shown to be associated with susceptibility to EBV-associated HL and NPC. We hypothesized that in patients carrying protective alleles, tumor cells need to downregulate HLA expression to escape killing by cytotoxic T cells. Vice versa, tumor cells in patients carrying risk allele do not have the pressure to lose HLA expression. In **Chapter 3 and Chapter 4**, loss of HLA expression was studied in the context of known risk and protective alleles.

Several HLA susceptibility alleles have been identified for cHL and EBV-stratified subgroups in previous studies[9,10]. This suggests a role for antigen presentation in the pathogenesis of cHL. In **Chapter 3**, we aimed to test our hypothesis that protective allele carriers have a higher pressure to lose HLA expression as compared to risk allele carriers. On one hand tumor cells need to retain expression of HLA to avoid killing by natural killer cells, while on the other hand tumor cells need to lose HLA expression to avoid killing by T cells. We conducted a meta-analysis to identify consistent risk and protective alleles in a combined Dutch and Scottish/North English cohort. HLA expression was examined in a combined cohort of 338 classical HL (cHL) patients and in EBV-stratified subgroups. Loss of HLA class I expression was observed in 72% of cHL patients, more frequently in female, EBV-negative cHL and nodular sclerosis subtype. cHL patients carrying the protective allele HLA-DRB1*07 lost HLA class II expression more frequent than non-carriers, consistent with our hypothesis. Similarly, EBV-negative DRB1*15/16 risk allele carriers retained HLA class I expression more frequently than non-carriers. In EBV-positive cHL patients, HLA-B*37 risk allele carriers retained HLA class I expression more frequently than non-carriers, consistent with our hypothesis. For other susceptibility alleles, HLA loss was either not significantly different between carriers and non-carriers, or the differences were significant but in opposite direction. In conclusion, loss or retention of HLA expression by tumor cells is associated with a subset of the protective or risk alleles, while not in others These data suggest that the susceptibility effects of HLA alleles are early events in pathogenesis, while the pressure on tumor cells to downregulate HLA is most likely related to emerging immune responses later on.

In **Chapter 4**, a study similar to the HL study was performed for NPC focusing on the unique Bidayuh population**.** The Bidayuh population is a minority ethnic subgroup in Malaysia with extreme high risk for NPC. To our knowledge, all large cohort studies

reporting the association of HLA with NPC have been conducted on the Chinese population residing in China or other countries. To date, there are no studies on association of HLA in the Bidayuh population. So, we performed HLA typing in this population to determine if Bidayuhs share the same risk or protective alleles with other high-risk populations. Due to great differences in allele frequencies of Bidayuh and Chinese, we did not find any association of previously identified risk or protective alleles to NPC in Bidayuh. On the contrary, three novel HLA alleles were found to have possible associations with NPC in Bidayuh. HLA-A*24:07 which is a dominant allele in the Bidayuh population, was identified as a protective allele in Bidayuh. Whereas HLA-A*24:02 and HLA-B*15:25 were identified as risk alleles in Bidayuhs. The allele frequency of HLA-A*24:02 is lower while the frequency of HLA-B*15:25 is higher in Bidayuh as compared to Chinese. These findings suggest that HLA type is important in susceptibility to NPC in Bidayuh, but that the underlying mechanisms are different from those in other high-risk populations. Differences might be related to genetic variants in non-HLA genes in the antigen presenting and host defense pathways[11–13] or in EBV strain and characteristics of viral exposure. The pathogenesis of NPC is clearly not one on one related to HLA genotypes, so other mechanisms must be involved. As a next step, we explored whether loss of HLA expression by tumor cells was associated with patients carrying susceptibility alleles. Bidayuh NPC patients carrying the HLA-A*24:02 risk allele more frequently retained HLA expression (100%) compared to those who did not carry the risk allele (16.7%). However, the number of cases available for these analyses was limited, so additional studies are required to confirm this finding. No difference in the frequency of HLA loss was observed for the other two susceptibility alleles.

Much effort has been made to determine suitable biomarkers for screening of high-risk populations. As the majority of NPC cases in high-risk regions are EBV positive, many studies explored the clinical utility of EBV serology tests as a screening tool. Recent studies showed that combination of two EBV serology tests, i.e., VCA IgA and EBNA-1 IgA, had a better performance than single serology tests in detecting NPC in the Southern Chinese population (AUC 0.97 in combined marker vs. 0.95 and 0.94 in individual marker)[14,15]. However the incidence of NPC in this seropositive subgroup was only less than 1%. Other studies demonstrated that plasma EBV DNA test alone could be used as a screening tool[16,17]. Re-testing EBV DNA load in 1,045 seropositive patients by means of nasopharyngeal swab were investigated for added value to screening programs in high-risk populations[18]. EBV DNA was detected in 89% of the 1,045 seropositive subjects, but only eight subjects were diagnosed with NPC. This study suggested that testing EBV DNA load in seropositive subjects could reduce the number of subjects that need close follow-up, although the overall reduction remains low. For developing countries, such a large-scale screening program is a considerable burden on the healthcare system and there is a need for improved tests to more accurately define the high-risk group.

Both EBV serology tests and EBV DNA test have been demonstrated to be useful as screening tools for NPC. More recently, other biomarkers such as plasma microRNAs have been reported for NPC. In **Chapter 5**, we evaluated the performance of six established biomarkers (i.e., four EBV serology tests namely EA IgA, EA IgG, EBNA-1 IgA, VCA IgA and two EBV DNA tests that differ in annealing site and amplicon size, namely BamHI-W 76 bp and EBNA1 99 bp) and four newly-reported biomarkers (i.e., new EBV DNA test BamHI-W 121 bp and plasma ebv-miR-BART7-3p, hsa-miR-29a-3p and hsa-miR-103a-3p). The goal was to evaluate the clinical utility of single or combinations of multiple biomarkers to detect NPC among populations of mixed NPC incidence rates, so that evidence-based strategies can be deduced for NPC screening and monitoring among populations of mixed NPC incidence rates. Among all 10 biomarkers, the EBV DNA test amplifying 76 bp of the BamHI-W region had the highest sensitivity to detect NPC in our cohort of 232 NPC cases. As expected, analysis of the larger fragment of the BamHI-W region (121 bp) had a lower sensitivity but a higher specificity as compared to the more routinely used BamHI-W 76 bp test. None of the miRNAs performed better than the EBV DNA-based tests. Lower areas under the curves (AUCs) were observed for all three miRNAs (range of 0.5071 - 0.7737) as compared to the EBV DNA tests (range of 0.9303 – 0.9832). To evaluate the performance of combinations of biomarkers to detect NPC, three different decision tree algorithms and two different validation strategies were evaluated, resulting in seven predictive models. Based on these models, it appears that the BamHI-W 76 bp test is essential for NPC detection as it is the only biomarker to be included in all models. Combined testing of BamHI-W 76 bp and EA IgG had improved sensitivity but reduced specificity. Meanwhile combination of BamHI-W 76 bp and VCA IgA had improved specificity at the expense of reduced sensitivity. The *EBNA1* DNA test was the best marker for prognosis of survival, also outperforming the BamHI-W 76 bp DNA test and was included in all decision tree models.

Collection of plasma for diagnostic or prognostic tests is considered much less invasive than acquirement of tissue biopsies. Besides plasma samples, we evaluated nasal washings (NW) which requires an even less invasive sampling method than collection of plasma. Our hypothesis was that testing material obtained from a site adjacent to the site at which NPC usually presents could increase the chance of capturing biomarkers shed from tumor cells. In **Chapter 6**, the levels of 27 miRNAs and EBV DNA in NW were evaluated as biomarkers for the detection of NPC. Both EBV DNA tests (BamHI-W 76 bp and *EBNA1* 99 bp) and seven out of 27 tested miRNAs showed significant different levels between NPC patients (n = 46) and non-NPC patients (n = 73, patients visiting ear, nose and throat (ENT) clinic without NPC or other cancers). All nine biomarkers showed AUC above 0.7. These biomarkers were subjected to logistic regression analysis to determine whether combinations of any of the biomarkers could predict NPC. The multiple logistic regression model with inclusion of *EBNA1* and hsa-miR-21 showed better performance in detecting NPC than any of the nine biomarkers alone. Follow-up of EBV DNA load in 14

patients over a period of 16 - 162 months to determine if NW EBV DNA could predict recurrence of NPC revealed reduced or lack of EBV DNA in eight patients that were in remission. Two patients with recurrent and residual NPC demonstrated elevated NW EBV DNA load over time. In the remaining four patients, one had initial spike of NW EBV DNA at three months before detection of lung metastasis and the NW EBV DNA load decreased over a period of about 10 months, one had complete remission but weak positive for NW EBV DNA, while the other two with recurrence or lung metastasis had decreasing/negative NW EBV DNA throughout follow-up. In total, NW EBV DNA tests showed a pattern consistent with the clinical situation for 10 out of 14 patients.

In **Chapter 7** and **Chapter 8**, the utility of cell-free DNA (cfDNA) as biomarker was explored in HL and PTLD, respectively. Tumor cells in HL biopsies represent less than 1% of the total number of cells. The scarcity of the tumor cells leads to difficulties in generating genomic profiles of the tumor cells. Furthermore, due to intra-tumor heterogeneity, a single biopsy might not be fully representative of the genomic aberrations present in the total tumor. In **Chapter 7**, copy number profiles of HL patients were generated using low-coverage whole genome sequencing (lcWGS). In addition, detection of EBV was carried out by targeted sequencing of BamHI-W and *LMP1*. Previous studies demonstrated that cfDNA fragments of tumor origin are shorter in length as compared to cfDNA originating from non-tumor cells. To improve CNV calling in our samples, in silico size selection was performed on our lcWGS data, which indeed increased the percentage of cases for which we could identify CNVs. This prompted us to focus on the CNV calling results obtained for the in silico size selection analysis. The average coverage after size selection was 0.04x in HL cfDNA samples. CNVs were detected in diagnostic plasma samples of 59% (26 out of 44) of classical HL (cHL) and in none of the six nodular lymphocyte predominant HL. In line with previous studies using cfDNAs[19,20] and purified HRS cells[21,22], the most frequent recurrent CNVs were 2p gain (34%), 9p gain (32%), 12q11-15 gain (23%) and 6q loss (20%). Although the frequency of gains and losses at each chromosomal region detected in our study was lower as compared to previous studies, probably due to suboptimal processing of plasma samples, we were able to identify the distinctive CNVs reported in HL. The recurrent CNV regions (i.e., 2p gain, 6q loss, 9p gain and 12q11-15 gain) included loci of known driver genes. An overview of the potential driver genes at these loci is given in Table 1. These genes play critical roles in the constitutive activation of signaling pathways known to be involved in the tumorigenesis of HL.

Comparison of the CNV profiles of EBV-positive with EBV-negative cHL demonstrated marked differences. This implicated different pathogenetic mechanisms driving EBV-positive and EBV-negative cHL. EBV-positive cHL has less dependency on the activation of NF-κB through the gain or loss of genes involved in this pathway, as evidenced by the lower frequency of 2p gain in EBV-negative cHL as compared to EBV-positive cHL. Mutual exclusivity of LMP1 overexpression and somatic aberrations of genes in NF-κB reported in

NPC[23] affirm that EBV-positive and EBV-negative tumors rely on different mechanisms for NF-κB activation. Targeted sequencing of the EBV BamHI-W and *LMP1* gene regions in cfDNA samples of HL patients revealed 100% sensitivity in detecting EBV-positive cHL patients. The median percentage of EBV reads in EBV-positive cHL patients were significantly higher than that of EBV-negative cHL. Further analysis of SNVs in the deep targeted sequencing data is ongoing.

Table 1. CNVs in loci involved in signaling pathway driving tumorigenesis of cHL.

Signaling pathway	Chromosomal region	Gain or loss	Number of cases with CNVs		Genes	Regulator
			EBV positive n = 6	EBV negative n = 19		
NF-κB	2p16.1 - 2p15	gain	1	13	REL	positive
NF-κB	17q21	gain	1	5	MAP3K14	positive
NF-κB	6q23.3	loss	0	9	TNFAIP3	negative
NF-κB	6p21.1	loss	0	6	NFKBIE	negative
JAK/STAT	9p24.1	gain	5	9	JAK2	positive
JAK/STAT	12q11 - 12q15	gain	2	8	STAT6	positive
JAK/STAT	17q21	gain	1	5	STAT3, STAT5A/5B	positive
Immune evasion	9p24.1	gain	5	9	CD274, PDCD1LG2	positive

The focus of the study described in **Chapter 8** was on monomorphic PTLDs, of whom 13 out of 18 patients were classified as diffuse large B-cell lymphoma (DLBCL) and the remaining patients as undefined. An approach similar to the approach for HL was applied on these samples, again focusing on CNVs and EBV. CNVs were detected in cfDNA samples of 61% (11 out of 18) of the patients. The most frequent CNVs were 3q26.2-29 gain, 6q11-21 loss, 11q14.1-25 gain and 18q21.1-21.33 gain. Similar to cHL, more genomic aberrations were observed in EBV-negative PTLD as compared to EBV-positive PTLD. The most frequent CNVs detected in PTLD of our study cohort represent those previously reported to be involved in the tumorigenesis of B-cell lymphoma. Genomic regions showing recurrent gains, i.e., 3q and 18q, include the *BCL6* and *BCL2* proto-oncogenes which are important for the regulation of proliferation and apoptosis of B cells. One of the four PTLD cases with 11q aberrations demonstrated gain of 11q23.3 and loss of 11q24.1. This specific chromosome 11 aberration was identified as a signature for a molecular variant of MYC translocation in PTLD patients that were sub-classified as Burkitt lymphoma (BL) patients[24]. The chromosome 11 signature was suggested to act as an alternative pathogenetic mechanism resulting in MYC activation in cases lacking the commonly observed MYC translocation. Re-analysis of the histology of the PTLD cases did not show a Burkitt like histology. We also observed gain of 11q without the loss of the

telomeric part of the q-arm, whether or not this is mutually exclusive with presence of MYC rearrangements remains unknown.

2. Future perspective

2.1. Screening strategies and early detection of NPC

Early detection of NPC can improve treatment outcome. In countries with high NPC incidence, population screening was carried out to allow diagnosing NPC in an early stage to reduce disease burden. Current screening strategies include testing first-degree family members of NPC patients or other selected populations with high incidence rate. Most studies were conducted with EBV serology tests and/or EBV DNA tests. One study demonstrated that family members of NPC patients often had higher EBV antibody levels than community controls with seropositivity in 81-97% first-degree family members while only 1-4% community controls were seropositive for the antibodies tested[25]. Another NPC screening study on first degree family members of NPC patients in Hong Kong detected NPC in 5/1,155 males (433/100,000 persons per year) and 7/1,404 (499/100,000 persons per year) females, which was much higher than the average annual NPC incidence during the time of the study period (24.1/100,000 and 9.6/100,000 persons per year in males and females, respectively. Of the subjects detected with NPC in this screening program, 41% had stage I disease, compared to only 2% in patients being referred to the hospital for treatment[26]. It would be interesting to investigate whether there is any relation between HLA alleles associated with NPC and EBV serology or EBV DNA tests. This could potentially be added in the screening program of first-degree family members of NPC patients to reduce the number of individuals indicated for follow-up. For individuals carrying protective alleles the follow-up could be reduced or completely stopped, while for individuals carrying risk alleles the frequency of follow-up could be increased.

The NPC incidence is remarkably high among the Bidayuh population in Malaysia. Most of this population resides in Sarawak, located at the Borneo Island. The infrastructure (including healthcare facilities) in some districts of Sarawak state is less developed than other states in Peninsular Malaysia. Most large hospitals with good facilities, i.e., availability of ENT specialists, laboratory test facilities, etc. are located in larger cities. This increases the challenge to conduct NPC screening among high-risk populations in Sarawak. Besides, residents in suburban or rural areas often delay seeking medical attention due to the lack of awareness in early detection and accessibility to healthcare facilities. In this instance, sampling of NW that requires minimal clinical setting and expertise of healthcare workers is potentially useful. In view of the higher susceptibility to NPC in a few native populations of Borneo Island including Bidayuh and other populations that were not included in this thesis (i.e., Iban and Kadazan), this approach could be more feasible. Further studies focusing on the effect of NPC risk factors such as dietary choices,

environmental exposures, EBV strain and genetic factors on Bidayuhs, as well as Ibans and Kadazans, in comparison to other NPC high-risk populations could elucidate the underlying causes of the high NPC incidence rate.

2.2. Molecular profiling of lymphoma

Utilizing next generation sequencing (NGS) technologies is becoming more feasible due to improvement of NGS technology and reduction in cost. NGS is valuable for genomic profiling to identify recurrent somatic aberrations and to gain insight into the pathogenetic mechanisms driving the tumor cells. In HL, in which the tumor cell percentage is less than 1%, obtaining sufficient tumor cell DNA from tissue biopsies to perform NGS is challenging. In this thesis, the potential of cfDNA as biomarker was explored. For treatment response evaluation, genomic aberrations detected in baseline cfDNA samples could be tracked in sequential cfDNA samples throughout the treatment and follow-up period. Recurrent hotspot mutations that are being detected in cfDNA samples at diagnosis could be tested with the highly sensitive droplet digital PCR (ddPCR) technique during follow-up. Due to limited amount of cfDNA especially when tumor load is low, multiplex ddPCR could be carried out to detect multiple mutations simultaneously to increase the reliability of the test result. Whether or not multiplex ddPCR can outperform NGS based detection of somatic mutations needs to be established. An advantage of NGS is that a broader target panel can be studied, but the disadvantage is that the variant allele frequencies are below the detection limit, depending on the amount of plasma available and the cfDNA yield, especially for minimal residual disease detection. The utility of other molecular detection tools could be explored in future studies. The clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas) system has recently gained wide attention. It binds to target DNA or RNA by recognizing distinct motifs called protospacer-adjacent motifs (PAM) guided by CRISPR, and then cleaves the target DNA or RNA with a Cas nuclease. Cas consists of different classes and subtypes of nucleases, each with a different mechanism. This system has been used for (i) detection of mutations and viruses in different cancers and (ii) directly editing the DNA or RNA transcripts of driver genes involved in oncogenesis.

The CRISPR/Cas system has been used for detection of mutations in several studies. One study used CRISPR/Cas9/Cas12a to increase detection of specific mutations in ctDNA by removing wild-type DNA from cfDNA samples. Removal of wild-type DNA was achieved by targeting them with guide RNAs and this resulted in reduce background signals, and thus increased sensitivity for the detection of the mutant DNA. The authors showed that, together with deep targeted sequencing, mutations in cfDNA of colorectal cancer patients were detected with high sensitivity and accuracy[27]. Other studies showed that CRISPR-Cas12a can sensitively detect *EGFR* (L858R and T790M) and *KRAS* (G12C) mutations from cell-free DNA and tumor, respectively[28,29]. It would be interesting to evaluate if mutation

detection in HL or PTLD using the CRISPR/Cas system has a higher detection sensitivity as compared to the commonly used NGS or ddPCR techniques.

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