Chapter 7

Summary, Discussion & Future perspectives
Classical Hodgkin lymphoma (cHL) is a B cell malignancy consisting of neoplastic Hodgkin Reed-Sternberg (HRS) cells, which generally comprise less than 1% of the total cell population, and a large majority of non-malignant reactive immune cells. This highly characteristic histology makes cHL a disease that is difficult to study. In our group we have a longstanding interest in the molecular, pathological and immunological background of cHL. In this thesis I focused on the tumor microenvironment, gene mutations, and the regulation of HLA expression.

**Microenvironment**

Primary tissue derived HRS cells cannot survive in culture, indicating that the tumor microenvironment is indispensable for tumor cell survival and growth. In chapter 2, we summarized the current knowledge on the cross-talk between the HRS cells and the reactive inflammatory cells. It is evident that the microenvironment in cHL is actively shaped and an essential component of the tumor. Both autocrine and paracrine signaling are involved in driving the activation, survival and proliferation of HRS cells. In addition, HRS cells shape the microenvironment by attracting cell types that are favorable for their survival and inhibiting cytotoxic cells that might induce anti-tumor immune responses. HRS cells manage to escape from an effective anti-tumor immune response via multiple mechanisms including loss of HLA class I and II molecules.

**Genetic landscape**

Using whole exome sequencing we defined the spectrum of sequence variations that commonly occur in HL cell lines in chapter 3. Overall, we identified 477 recurrently mutated genes in HL regardless of subtype and 382 recurrent mutations specifically in cHL. Within this list of consistently mutated genes, cell adhesion was the most common gene ontology. This is quite surprising, as mutations in this group of genes have not been reported previously. The significance of the mutated genes was further strengthened by the marked overlap with copy number gain or loss regions and with genes showing a significantly differential expression pattern. A high proportion of the genes in this latter group belonged to the chromatin modification and transcription gene ontology group. Three genes with truncating mutations in part of the Hodgkin lymphoma cell lines showed an enhanced expression in comparison to germinal center B cells. This finding was unexpected, as presence of nonsense mutations may lead to RNA destabilization and lower
levels. For MYB the enhanced levels might be related to the low miR-150 levels as previously reported in cHL [1]. MiR150 targets MYB in normal B cells in a dose-dependent manner and thereby dramatically affects lymphocyte development and response [2, 3]. In Hodgkin lymphoma, the low miR-150 levels may thus be responsible for the enhanced MYB mRNA levels despite the presence of truncating mutations. The mutational landscape in Hodgkin lymphoma has several genes in common with other lymphoma subtypes, especially with diffuse large B cell lymphoma and follicular lymphoma. Overlapping genes included, amongst others, chromatin modification and transcription genes. Our data support a possible role of these genes in HL pathogenesis and will help to select candidate genes for further validation by Sanger sequencing and for confirmation in primary HRS cells.

**Regulation of HLA expression**

The human leukocyte antigen (HLA) is a crucial component of the human immune system. Both HLA class I and class II are critical in the interaction between tumor cells and components of the innate and adaptive immune system. HLA class I restricted CD8+ cytotoxic T-cell (CTL) responses are known to target EBV and HPV infected cells through cell-mediated immunity [4]. β2-m is an essential component of the HLA class I-antigen complex and is needed for expression on the cell membrane [4]. HLA class II restricted CD4+ cells control the EBV infection and malignant transformation *in vitro* [5]. Changes in HLA expression have been identified in many malignancies and tumor cells have been shown to use a large number of strategies to achieve HLA downregulation. These include loss of the HLA loci, loss of β2-m, loss of regulatory factors, epigenetic changes and gene mutations. In this thesis we focused on HLA downregulation at different levels, namely identification of gene mutations in *HLA* or *HLA* associated genes and the role of PML and SATB1 in the regulation of HLA expression.

**HLA class I**

In Chapter 4 we showed a normal HLA class I membrane expression in 40% of EBV+ and 20% of EBV- cHL cases. There was a stronger than normal HLA class I protein expression in approximately 40% of EBV+ cHL and not in EBV- cHL patients. HLA class I negative cases were observed in 20% of the EBV+ and in 80% of the EBV- cHL cases. These findings are quite remarkable as proper expression of HLA class I-antigen complexes on EBV+ HRS cells should result in effective
EBV-specific anti-tumor immune responses. Previous studies from our group showed a strong association of two HLA-A types with EBV+ cHL [6, 7]. HLA-A*01 is a risk type and has a low affinity for LMP1 and LMP2 derived antigenic peptides, whereas HLA-A*02 is a protective type that has a relatively high affinity for LMP1 and LMP2 derived antigenic peptides [8, 9]. Thus, lack of the HLA-A*02 type might partially explain why EBV+ HRS cells with HLA class I expression are not recognized and targeted by CTL mediated anti-tumor responses. In addition, HRS cells express immunosuppressive cytokines, such as TGF-β and IL-10, which can inhibit the cytotoxicity of these CTLs [10, 11]. Whether EBV+ HRS cells would benefit from an even increased HLA class I expression remains an open question. In contrast to the frequent retention of HLA class I in EBV+ cHL, HLA class I expression was more frequently observed in HPV- oropharyngeal squamous cell carcinoma (OpSCC) as compared to HPV+ cases. This frequent loss of HLA in OpSCC fits with the concept that HLA loss provides an escape from CD8+ T cell mediated cytotoxic anti-viral immune responses. In ovarian cancer, 27% of the cases showed a negative HLA class I staining, 39% a partially positive and 34% a positive staining. HLA class I downregulation and defects of antigen processing components (TAP1 and TAP2) are independent markers for adverse prognosis in ovarian cancer, which indicates that immune escape occurs in a proportion of patients and predicts poor clinical outcome [12-14].

A quite recently established mechanism of HLA regulation is chromatin remodeling. PML and SATB1 are two major chromatin-remodeling proteins that have been shown to regulate HLA class I in some normal cell types, but have not been studied much in malignancies. We studied expression of PML and SATB1 in cHL, squamous cell carcinoma from the head and neck region and ovarion adenocarcinoma (Chapters 4-6). PML is the main component of nuclear bodies (PML-NBs) that can be detected as small discrete intranuclear dots by immunohistochemistry. A direct interaction between SATB1 and PML in -NBs is necessary for regulation of the chromatin-loop organization, including the HLA loci. They are directly associated with matrix attachment regions (MAR) in chromatin loops and upstream regulatory sequences of genes within the HLA class I locus [15].
We did not observe correlations between PML, SATB1 and HLA class I in EBV- cHL cases, indicating that other mechanisms should be involved. We did demonstrate that a stronger than normal class I expression is positively correlated with the number of PML nuclear bodies and negatively with the percentage of SATB1 positive HRS cells in EBV+ cHL (Chapter 4), indicating a possible causative association. In addition to cHL, we also studied expression of these genes in two solid tumor types. The average number of PML-NBs was not correlated with HLA class I staining in OpSCC or HPV stratified OpSCC. Also the SATB1 staining was not correlated with HLA class I staining in total as well as in HPV stratified cases (Chapter 6). Thus, despite the presence of oncogenic viruses in both EBV+ cHL and HPV+ OpSCC malignancies, the HLA expression and the possible regulation via PML and SATB1 are quite different. The second solid tumor type we studied was ovarian cancer (Chapter 6). This cancer type is not associated with a virus. The average number of PML-NBs was positively correlated to the level of HLA class I expression, similar to the effect we observed in EBV+ cHL.

These striking differences suggest that different mechanisms are involved in the down- or upregulation of HLA in these different types of tumors. In cHL we scored HLA based on membrane positivity, whereas in the two solid tumors we scored the overall positivity irrespective of cellular location. As exclusive cytoplasmic staining is rare in these tumor types, the impact of the differences in scoring will only have a limited effect on the overall results. A second potentially complicating factor might be the differences in staining patterns. In cHL, we showed enhanced staining, normal staining and complete absence of HLA class I staining in cHL, which was based mainly on the comparison with directly neighbouring cells. In ovarian cancer and OpSCC complete loss of HLA class I, partial loss and normal HLA class I staining was based on the percentage of positive cells. The partial positive OpSCC cases were characterized by loss of HLA class I expression in differentiating areas of the tumor, a pattern that is similar to the pattern in normal squamous epithelium. Despite the lack of significant associations in OpSCC, the partly positive staining pattern was consistent with the increased number of PML-NBs, indicating a putative role of PML in regulation of HLA expression in differentiating squamous cells, which might be lost in a later stage during tumor progression. Based on our combined results, it can be speculated that mechanisms of HLA expression regulation differ in different cancer cell types.
The results obtained so far support a possible role of PML-NBs and/or SATB1 in HLA class I regulation in different malignancies. A disadvantage of our approach is that we used one antibody that recognizes all PML isoforms and also one antibody that detects all three classical HLA class I genes. Therefore, we further analyzed a possible association of these two genes and separate HLA class I genes at the mRNA level in cHL cell lines. Significant positive correlations were found between PML-III and HLA-C and between PML-III and β2M (Figure 1). Suggestive, though not significant, correlations were observed between various PML isoforms and several HLA genes and between SATB1 and some HLA genes (data not shown). The limited number of available cell lines precluded more definitive conclusions. Functional studies on cell lines with enhanced or reduced expression of SATB1 or PML are required to prove true interactions.

Figure 1. Significant positive correlation between PML-III and HLA-C as well as between PML-III and β2-m at mRNA level in 6 cHL cell lines.
A potential other mechanism causing loss of HLA class I expression in HL was obtained from our whole-exome sequencing data. We identified mutations in the ATG start codon of the \textit{B2M} gene in L428 and DEV, which explains the lack of membrane β2-m and HLA-class I in these two cell lines (own unpublished data). A frame-shift mutation in \textit{HLA-A} was identified in SUPHD1. The lack of HLA-A specific antibodies precluded a further analysis at the protein level. To the best of our knowledge there are no reports on \textit{B2M} mutations in ovarian cancer or OpSCC cases.

**HLA-class II**

A normal HLA class II expression pattern was observed in 50% of EBV+ and 50% of EBV- cHL cases, with a stronger than normal HLA class II expression only in four EBV+ patients and one EBV- cHL patient. In contrast to the HLA class I staining, we did not see a difference in HLA class II loss in relation to EBV in cHL. This indicates a putative different mechanism for HLA class I and class II regulation. The number of PML-NBs and the percentage of SATB1 positive cells were not correlated with classical HLA class II expression in EBV+, EBV- or the total cHL patient group (Chapter 5). A disadvantage of our approach is that we analyzed general PML staining (including all PML isoforms) and we also analyzed general classical HLA class II staining (including HLA-DR, HLA-DP and HLA-DQ). To study isoform specific effects in relation to individual HLA class II genes (both classical and non classical) we further investigated the mRNA expression levels in six HL cell lines. A significant positive correlation was found between PML-V and HLA-DMA. PML-V also showed borderline significant positive association with HLA-DPB1 and the class II transactivator CIITA. PML-IV was borderline significantly associated with HLA-DOB. Suggestive correlations were observed between various PML isoforms or SATB1 and several HLA genes. Again, the limited number of cell lines precluded more definitive conclusions, but do support a potential association. To show a functional role of SATB1 in regulation of HLA class II expression, we inhibited SATB1 in the L1236 cHL cell line using shRNA-based knockdown. One of the two SATB1 shRNAs induced a significant SATB1 reduction both at the SATB1 mRNA level and protein level. Inhibition of SATB1 by this shRNA induced a significant upregulation of HLA class II expression (Figure 2). These ongoing experiments support a role of SATB1 in enhancing HLA class II expression upon inhibition of SATB1 in L1236 cells. The regulation by PML is complex with seven different
isoforms that appear to have different functions with respect to HLA regulation. Functional experiments testing inhibition of specific PML isoforms and pan-PML are ongoing.

Figure 2. The effect of SATB1 shRNAs on HLA class II expression in L1236. A) Western blot showing the inhibition of SATB1 by shRNAs. The SATB1 shRNA1 induced a ~80% reduction and SATB1 shRNA2 showed a ~25% reduction at the protein level. Actin was used as internal control. B) FACS staining results showing mean fluorescence intensity shift of GFP+ cells compared with GFP- cells normalized with scramble to indicate the effect of HLA class II expression in GFP+ cells with SATB1 shRNAs. Inhibition of SATB1 by shRNA1 significantly upregulated HLA class II expression (P=0.02). Inhibition of SATB1 by shRNA2 showed no significant effect on HLA class II expression.

Whole exome sequencing of the HL cell lines revealed only one missense mutation in HLA-DRB1 in SUPHD1. Protein expression of HLA-DRB1 has not been determined as there are no specific antibodies. In the other cell lines and genes, no mutations in HLA class II genes have been observed. Another gene of interest in the regulation of HLA expression is CIITA. Data from the Broad institute combined with our own exome sequencing data indicated presence of CIITA mutations in four of the 7 HL cell lines, i.e. L428, L540, L1236 and DEV. In addition, it was already shown that this gene is inactivated via a chromosomal translocation in KMH2 [16]. CIITA encodes for the HLA class II transactivator, which is the master regulator of HLA class II expression. Inactivation of this gene might thus contribute to downregulation of the HLA class II genes. HLA class II is negative in DEV cells and positive in other cell lines (Chapter 5 and data not shown). This indicates that the CIITA mutation in DEV may lead to loss of expression of multiple HLA class II genes.
(DP/DQ/DR), whereas functional consequences of the CIITA mutations in the other cell lines remain unknown.

Overall, we showed involvement of different mechanisms in the regulation of HLA expression (Table 1). Enhanced expression of HLA class I might be related to SATB1 loss. Gain of the number of PML-NBs in EBV+ cHL and the number of PML-NBs was associated with HLA class I in ovarian cancer, but not in OpSCC. The complete loss of HLA class I in HL might be related to gene mutations affecting β2-m or the classical HLA genes themselves. For HLA class II the associations at the mRNA level, combined with the functional studies with the shRNA constructs, support a role of SATB1 and PML in regulating HLA class II in HL. In addition to these effects, genetic aberrations of CIITA might further contribute to the HLA class II loss. Another mechanism frequently reported in non-Hodgkin lymphoma, i.e. loss of the 6p21 region containing the HLA gene loci, might also contribute to the loss of HLA in HL as we found losses in this region in four of the seven cell lines (Chapter 3).

Table 1. Possible mechanisms involved in the regulation of HLA expression based on our data as well as data in the literature.

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√ indicates possible relevance based our study or based on the literature; --- indicates no proof for an association; NK indicates not known.
PML and SATB1
An interesting observation in these studies was that the average number of PML-NBs was quite different between the different tumor types. For cHL and OpSCC, we tested whether the number of PML-NBs per cells was dependent on the size of the tumor cells. The surface area of HRS cell nuclei in 4 μm tissue sections ranged from 66 to 412μm² and the number of PML-NBs per nucleus ranged from 0 to 40. The number of PML-NBs was not related to an increase in the size of the HRS cell nuclei. In ovarian cancer cells 0 to 19 PML-NBs were observed per tumor cell nucleus (surface area 35 to 224 μm²) and no significant correlation was observed between the size of the tumor cell nucleus and the number of PML-NBs. In OpSCC, the average number of PML-NBs varied from 0 to 13 per tumor cell nucleus in a 4 μm thick tissue section. Based on the findings in HL and ovarian cancer we did not further explore a relation with the tumor cell size. Overall, we did not see a correlation between cell size and the number of PML-NBs. Nevertheless, we did observe more PML-NBs in HRS cells, which are on average larger than the tumor cells of OpSCC and ovarian cancer.

In cHL, about half of the cases stained positive for SATB1. In OpSCC, only 10% of all cases stained positive for SATB1 and this percentage was similar for HPV+ and HPV- cases. SATB1 expression was more common in HRS cells compared with tumor cells of OpSCC and ovarian cancer. We did not study SATB1 expression in ovarian cancer because in the test phase all cases analyzed were negative. In contrast to our findings that SATB1 was usually negative in ovarian cancer, two recent papers did find SATB1 expression in a high proportion of the cases [17, 18]. In both studies the authors used a different antibody than we did, which might explain the difference. We have to analyze this antibody on our cases to clarify this difference.
**Future perspectives**

To complete the exome-sequencing work, we need to select candidate genes to validate the mutations in primary HRS cells. Potential cancer-driving genes will be taken into consideration if the genes: 1, are the most frequently mutated in HL cell lines, 2, harbor nonsense mutations which may yield a truncated protein product, 3, are located in chromosomal regions with copy number loss and downregulated expression in cHL vs GCB and/or 4, are of particular interest like HLA and related genes. In addition, we need to determine the protein expression of candidate genes in HRS cells by immunohistochemistry, especially for genes harboring truncating mutations, immunostaining might represent a straightforward approach to screen primary tissue samples. Most importantly, we should establish the functional role of selected genes in HL pathogenesis, by up- or downregulating these genes and studying the induced phenotype changes. These changes could be related to malignant transformation and/or escape from anti-tumor immune responses.

So far, there is no clear link with \textit{CIITA} gene mutations and HLA class II protein expression. In some studies it has been shown that knockdown of pan-PML by shRNA inhibited IFN-\( \gamma \)-induced HLA class II and CIITA in a cervix carcinoma cell line and human primary fibroblasts [19, 20]. Based on these papers, we can speculate that the effect of CIITA inactivation will become visible only upon activation of the HL cells. To investigate this we should study if the cells carrying mutated \textit{CIITA} genes are able to enhance HLA class II expression upon IFN-\( \gamma \) treatment. We expect lower HLA class II induction in the cell lines with inactivating \textit{CIITA} mutations.

Many other mechanisms can be involved in downregulation of HLA expression. Since our group is interested in the role of specific HLA alleles and haplotypes, it is worthwhile to associate HLA genotyping with HLA expression. It has been shown that HLA-A*01 was significantly overrepresented in EBV+ cHL patients compared with EBV- cHL and controls [6]. In contrast, HLA-A*02 was less common in EBV+ cHL patients [6]. It can be speculated that certain HLA types are unfavorable for HRS cell survival, and require downregulation during the malignant transformation, whereas other alleles are favorable or not harmful and do not require downregulation for tumor cell survival. Unfortunately, we did not have enough power to explore if HLA loss is related to specific HLA typings, since we did not
Summary, discussion and future perspectives

have HLA typing data for a substantial part of the patients in our study (Chapter 4). In our GWAS and HLA typing studies we explored differences between EBV+ and EBV- cases, but not between HLA class I positive and negative cases. This might still be interesting to do, although the strong association between these two factors might complicate the analysis. The HLA class II expression is not so strongly associated with EBV, and we previously found that three microsatellite markers mapping in the HLA class II locus were associated with HLA class II expression [21]. It would be of interest to further extend these analyses to the HLA class II typing data and the GWAS data and explore putative associations with HLA class II expression.

We observed positive correlations between different PML isoforms and multiple HLA genes at the mRNA level. Because different PML isoforms show different associations [15, 19, 20, 22, 23], we need to identify the role of specific PML isoforms and pan-PML. These experiments are ongoing in HL and should be extended to solid tumor types studied in this thesis. Putative effects of inhibiting SATB1 or PML isoforms on HLA class I and II gene expression should also be determined by qRT-PCR, allowing analysis of individual HLA genes. Based on the HLA typing data of the HL cell lines, we might be able to screen some specific HLA alleles also by FACS depending on the availability of specific antibodies. Based on the studies that show the effect of pan-PML by shRNA on IFN-γ-induced HLA class II and CIITA expression [19, 20], we should also study the role of specific PML isoform shRNA constructs on the regulation of CIITA and HLA class II expression upon IFN-γ treatment.
**Solid tumors**

In our OpSCC series we only had 21 HPV+ cases, which might not have been enough to establish a correlation. We should expand this series of HPV+ OpSCC cases. Functional experiments with overexpression and / or shRNA constructs can be done to further support a direct functional link. We found that in a proportion of OpSCC cases, the smaller, least differentiated tumor cells were HLA class I positive and had the highest numbers of PML-NBs. In contrast, both HLA class I expression and the numbers of PML-NBs in differentiating tumor cells were lower or absent. This HLA pattern is similar to what can be observed in squamous epithelium and skin tissue [24-26]. Consistent with our observation in tumor cells, we observed that the loss of HLA class I also occurred together with the loss of PML-NBs in these normal cells (data not shown), which indicates that PML may play a role in the regulation of HLA class I expression during the keratinization stages in both tumor cells and normal squamous cells. We need to further characterize PML and HLA class I expression in differentiated and undifferentiated tumor cells and normal squamous cells to explore a possible role of PML in HLA regulation upon differentiation. For ovarian cancer, we should test the other SATB1 antibodies and establish a possible association with HLA class I expression.
References


Nederlandse samenvatting
Het klassieke Hodgkin lymfoom is een vorm van lymfklierkanker die ontstaat uit de B lymfocyten. Het aangedane weefsel bevat een zeer laag percentage tumorcellen, de zogenaamde Hodgkin Reed-Sternberg (HRS) cellen, die zich bevinden in een omgeving die bestaat uit een grote overmaat aan normale reactieve immuuncellen. Deze samenstelling is uniek voor het Hodgkin lymfoom en het lage aantal tumorcellen maakt het moeilijk om deze cellen te onderzoeken. In onze onderzoeksgroep staan de moleculaire, pathologische en immunologische aspecten van het Hodgkin lymfoom centraal. In dit proefschrift heb ik onderzoek gedaan naar de karakteristieke micro-omgeving van de tumor cellen, genetische afwijkingen in de tumor cellen en de regulatie van de expressie van de antigeen presenterende HLA moleculen.

De micro-omgeving
HRS cellen uit primair patiënten weefsel kunnen niet overleven in *in-vitro* kweek systemen en dit geeft aan dat de tumorcellen de omliggende cellen nodig hebben voor overleving en groei. In *hoofdstuk 2* hebben we een uitgebreide literatuurstudie gedaan om de huidige kennis over de rol van de reactieve cellen in de micro-omgeving van de tumor cellen in kaart te brengen. Signalen vanuit de tumorcellen en de reactieve cellen dragen bij aan de activatie, overleving en groei van de HRS cellen. Daarnaast trekken de HRS cellen juist specifieke cellen aan die gunstig zijn voor hun overleving en onderdrukken ze de effectiviteit van cellen die een anti-tumor respons kunnen induceren. Herkenning door het immuunsysteem wordt daarnaast in een deel van de gevallen ook voorkomen door de eliminatie van de HLA moleculen. Op basis van deze literatuur studie is het duidelijk dat de samenstelling en activiteit van de reactieve cellen sterk wordt gestuurd door de tumorcellen en dat deze reactieve cellen een essentiële component van de tumor vormen.

Genetische afwijkingen
Om inzicht te krijgen in de genetische afwijkingen in HRS cellen, hebben we de next-generation sequencing technologie gebruikt om alle eiwit coderende genen te screenen op de aanwezigheid van mutaties. In *hoofdstuk 3* is te zien dat we in 7 Hodgkin lymfoom cellijnen 477 genen hebben geïdentificeerd die in tenminste 2 cellijnen een mutatie hadden. Een deel van deze gemuteerde genen ligt in
chromosomale gebieden waarin we verlies of winst van chromosomaal materiaal zagen. Daarnaast was er een aanzienlijke overlap met genen die differentieel tot expressie komen in Hodgkin lymfoom cellijnen t.o.v. normale B cellen. Deze bevindingen ondersteunen de relevantie van deze gemuteerde genen voor de pathogenese van Hodgkin lymfoom. Net als bij andere lymfoom types zagen wij dat de functies van de gemuteerde genen vaak geassocieerd zijn met chromatine modificaties en met immuun functie, zoals de HLA moleculen (zie hieronder). Onze data laten zien dat het mutatie patroon in Hodgkin lymfoom een aanzienlijke overlap heeft met andere lymfomen maar dat er daarnaast ook een groot aantal Hodgkin lymfoom specifieke mutaties aantoonbaar zijn. Er is verder onderzoek nodig naar de relevantie van deze genmutaties.

**Regulatie van HLA expressie**

De HLA moleculen vormen een essentieel onderdeel van het humane immuunsysteem en zijn verantwoordelijk voor het presenteren van intracellulaire (HLA klasse I) en extracellulaire (HLA klasse II) antigeen peptide aan de immuuncellen. Op deze manier kan het immuunsysteem reageren op virus en bacterie infecties en op tumorcellen. Beide HLA subklassen zijn van belang voor de interactie tussen HRS tumorcellen en reactieve cellen. Het is voor de tumorcellen gunstig om expressive van de HLA moleculen te verliezen, zodat ze niet herkend worden door het immuunsysteem en daarmee kunnen ontsnappen aan een anti-tumor response. Er zijn verschillende manieren om verlies van HLA expressie te induceren, namelijk verlies van de HLA genen op chromosoom arm 6p, verlies van β2-microglobuline, essentieel voor membraan expressie van HLA klasse I moleculen, verlies van regulerende factoren, gen mutaties en epigenetische veranderingen. In *hoofdstuk 4-6* hebben we de rol van PML en SATB1 bestudeerd in relatie tot HLA expressie in Hodgkin lymfoom, hoofd hals tumoren en in ovarium kanker. De eerste twee kankersoorten worden gekarakteriseerd door de aanwezigheid van een oncogeen virus, EBV bij hodgkin lymfoom en HPV bij hoofd hals tumoren, in een deel van de patiënten. Dit geeft ons de mogelijkheid om te kijken of verlies van HLA klasse I geassocieerd is met de aanwezigheid van een virus en dus de aanwezigheid van virale antigeen peptiden. Daarnaast hebben we onze next generation sequence data specifiek geanalyseerd op de aanwezigheid van mutaties in genen die geassocieerd zijn met antigeen presentatie en expressie van de HLA moleculen.
**HLA klasse I**

In hoofdstuk 4 toonden we normale HLA klasse I expressie in 40% van de EBV+ en 20% van de EBV- Hodgkin lymfoom patiënten aan. In 40% van de EBV+ gevallen zagen we een expressie sterker dan normaal en in 20% was de expressie volledig afwezig. In EBV- patiënten zagen we geen verhoogde expressie en verlies van expressie in 80% van de patiënten. Deze bevindingen waren opmerkelijk aangezien we juist in de EBV+ groep verlies van HLA klasse I hadden verwacht.

In tegenstelling tot onze resultaten bij Hodgkin lymfoom, vonden we verlies van HLA klasse I expressie voornamelijk in de HPV+ hoofd hals tumoren. Deze bevindingen zijn consistent met de hypothese dat verlies van HLA gunstig is voor het ontsnappen aan een effectieve anti-tumor immuun respons. In ovarium kanker vonden we verlies van HLA klasse I in 27% van de patiënten, gedeeltelijk verlies in 39% en een normale expressie in 34% van de patiënten.

Vervolgens bestudeerden we de associatie tussen HLA klasse I verlies en expressie van twee eiwitten die de expressie van HLA kunnen beïnvloeden door het veranderen van de chromatine organisatie in de celkern. In EBV- Hodgkin lymfoom patiënten vonden we geen associaties met PML en SATB1 expressie. In EBV+ Hodgkin lymfoom vonden we een positieve associatie met PML en negatieve associatie met SATB1 (Hoofdstuk 4). Dit was consistent met de eerder gepubliceerde resultaten waarin PML de expressie van HLA klasse I verhoogt en SATB1 de expressie van HLA verlaagt in andere celtypen.

In hoofd hals tumoren vonden we geen enkele associatie tussen HLA klasse I expressie en de expressie van PML of SATB1 (hoofdstuk 6). In tegenstelling tot de associatie in de EBV+ Hodgkin lymfoom groep vonden we geen associatie in de HPV+ hoofd hals tumoren. In ovarium kanker vonden we wel een positieve associatie tussen HLA klasse I en PML. We hebben SATB1 niet onderzocht in deze tumoren omdat we in de test fase geen enkele positieve tumor hebben kunnen aantonen (hoofdstuk 6). Op basis van deze opmerkelijk verschillen in associaties kunnen we concluderen dat in verschillende kanker types verschillende mechanismen betrokken zijn bij verlies van HLA klasse I expressie.

Een van de mogelijke andere mechanismen is verlies van HLA expressie door mutaties in de HLA genen of genen die direct betrokken zijn bij antigeen
presentatie. In Hodgkin lymfoom vonden we een aantal mutaties (hoofdstuk 3) die verlies van HLA klasse I expressie konden verklaren. Een mutatie in het start codon van β2-microglobuline werd gevonden in twee cellijnen, en een frame shift mutatie in HLA-A in een derde cellijn. Op basis van deze bevindingen kunnen we concluderen dat genmutaties kunnen bijdragen aan het verlies van HLA klasse I expressie.

**HLA klasse II**

In aanvulling op de HLA klasse I studies hebben we in Hodgkin lymfoom ook een analyse gedaan voor de HLA klasse II expressie en de mogelijk associatie met PML en SATB1 (Hoofdstuk 5). Een normaal HLA klasse II aankleuringspatroon konden we aantonen in zowel 50% van de EBV+ als de EBV- patiënten. Echter in tegenstelling tot de resultaten voor HLA klasse I, vonden we geen associaties van HLA klasse II met PML of SATB1. Een mogelijke verklaring hiervoor kan zijn dat we met de kleuringen tegelijkertijd naar alle isovormen kijken, dit zijn drie eiwitten voor HLA klasse II, HLA-DR, DP en DQ, en zeven isoformen voor PML, I t/m VII. Om meer specifiek inzicht te krijgen in een mogelijke associatie hebben we voor de Hodgkin lymfoom cellijnen ook gekeken naar associaties tussen de mRNA transcript levels van de individuele genen. We vonden op basis van deze analyse een significante associatie tussen PML-V en HLA-DMA, een borderline significantie tussen PML-V en HLA-DPB1 en de class II transactivator CIITA en een borderline significantie tussen PML-IV en HLA-DOB. Verder zagen we voor de meeste combinaties een trend in de verwachte richting, maar niet significant, waarschijnlijk doordat het aantal Hodgkin lymfoom cellijnen te beperkt was. Aanvullende functionele experimenten zijn nodig om te bewijzen of er wel of niet een regulerend effect is van PML of SATB1 op HLA klasse II expressie.

Op basis van onze eigen sequentie analyse data en de data gepubliceerd door het Broad instituut, kunnen we concluderen dat ook voor HLA klasse II expressie genmutaties een deel van het verlies kunnen verklaren. In één cellijn zagen we een mutatie in HLA-DRB1. Daarnaast zagen we puntmutaties in CIITA in vier van de cellijnen, terwijl er al een inactiverende chromosomale translocatie was beschreven in een vijfde cellijn. CIITA speelt een belangrijke rol bij de regulatie van HLA klasse II expressie, en inactivatie van dit gen kan heel goed leiden tot verlies van HLA klasse II expressie.

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