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## Expression of human leukocyte antigens in diffuse large B cell lymphomas

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### Citation

Riemersma, S. A. (2006, March 28). *Expression of human leukocyte antigens in diffuse large B cell lymphomas*. Retrieved from <https://hdl.handle.net/1887/4348>

Version: Corrected Publisher's Version

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**Note:** To cite this publication please use the final published version (if applicable).

# Summary

According to the recent WHO classification, diffuse large B cell lymphoma (DLBCL), the most frequent type of non-Hodgkin lymphoma, represents one disease entity as pathologists were not able to reliably distinguish between subentities on morphological grounds alone. However, DLBCL most certainly comprise several disease entities as they show remarkable heterogeneity with respect to presentation, response to treatment and dissemination pattern. Nearly 40% of the DLBCLs primarily present at extranodal sites of which the gastrointestinal tract is the most common (~30%). A minority of these lymphomas present at immune-privileged sites such as the CNS and the testis. Lymphomas growing at the latter sites are thought to experience growth advantage as they are more or less protected from cytotoxic T cell attack as local immune surveillance is hampered by the specific micro-environment and the blood-organ barriers. However, the immune-privileged sites are not completely devoid of immune responses, as rejection of tumours has been described, as well as the presence of small numbers of T cells under physiological circumstances. Loss of HLA class I expression as a mechanism to evade anti-tumour immune responses, has been described in many different types of solid tumours as well as in certain haematological malignancies. In the studies presented in this thesis, loss of HLA class I and II expression and the underlying mechanisms were investigated in extranodal DLBCLs that primarily presented in the CNS and testis, and compared to DLBCLs of lymph nodes and of other extranodal sites such as the stomach and skin. Moreover, the distribution of HLA-DR and DQ alleles was determined in a large group of testicular DLBCLs, and compared to a group of nodal DLBCLs and a group of healthy controls.

Previously, loss of HLA class I and II expression was reported to be correlated with worse prognosis in a small proportion of B cell lymphomas. In these reports no distinction was made between nodal or extranodal lymphomas apart from a group of aggressive lymphomas derived from the mediastinum. In **chapter 2** we studied HLA class I and II expression in nodal DLBCLs and 2 groups of extranodal DLBCLs from immune privileged sites: the testis and the CNS. We applied immunohistochemical staining on formalin-fixed paraffin-embedded tissue sections using a panel of mouse monoclonal antibodies (mAbs) directed against HLA-A, transporter-associated with antigen processing (TAP1) and HLA-DR and a rabbit polyclonal Ab directed against Beta-2-microglobulin (B<sub>2</sub>M). If fresh-frozen tumour material was available, we additionally stained for pan-class I (HLA-A,-B,-C in complex with B<sub>2</sub>M) and HLA-DQ. Loss of HLA-A expression was observed in 61% of the testicular, 55% of CNS lymphomas and 10% of the nodal lymphomas. Complete loss of expression as assessed by the pan-class I Ab was seen in comparable numbers of cases. Loss of class I could be explained in approximately half of the cases by concomitant loss of B<sub>2</sub>M or TAP1 expression. We probably even underestimated the number of cases with class I

aberrations as we did not study loss of specific class I alleles. This was not feasible as we did not have the required normal material to perform DNA typing of the class I alleles and because allele specific mAbs were not available to apply on formalin-fixed paraffin-embedded tissue sections.

For HLA class II, the differences between the nodal and the extra-nodal lymphomas were even more remarkable with 61% of the testis and 46% of the CNS lymphomas compared to 5% of the nodal lymphomas showing complete loss of HLA-DR expression. To investigate whether genetic defects at the HLA-region at chromosome 6p21.3 contributed to loss of HLA expression, we did loss of heterozygosity (LOH) analysis using a large set of polymorphic microsatellite markers specific for this region. The testicular and CNS lymphomas but not the nodal lymphomas showed allelic imbalance at high frequency, with nearly 70% of the cases showing LOH in the class III region at a marker 5 kb telomeric of TNF- $\alpha$ . However, three testicular cases showed a very interesting LOH pattern with retention of marker D6S1666 at the HLA-DR/DQ region and LOH at the directly flanking markers. This pattern was very suggestive of a small homozygous deletion with the remaining signal being derived from tumour-contaminating normal cells. To address this issue, we applied two-colour fluorescence in situ hybridization (FISH) on interphase nuclei of all cases with available frozen tumour tissue (i.e. 14 testicular, four CNS and seven nodal lymphomas). Three PAC probes specific for the HLA-DR/DQ region were used in combination with a probe for centromere 6. Ten normal tonsils were used as controls. PAC93N13 containing the DRB1 and DQA1 genes was homozygously lost in 8 of 14 testicular, in 2 of 4 CNS and in none of the nodal lymphomas. The PAC covering DRA was homozygously lost in 50% of the testicular and CNS lymphomas. The percentage of tumour cells showing the homozygous deletion varied between 7.0 and 71.5% which might reflect tumour heterogeneity and/or the presence of many infiltrating normal cells. The use of DNA-fiber FISH enabled us to exactly map the size of the deletions. The smallest homozygous deletion was approximately 100 kb and always included the HLA-DQA1 and HLA-DRB1 genes. Additionally we used 3 cosmid probes specific for the centromeric end of the class II (HLA-DPB), the class III (TNF- $\alpha$ , Lymphotoxin A) and the class I region (HLA-A). We found large hemizygous deletions of the class III and I region in 50% of the extra-nodal lymphomas.

Loss of heterozygosity (LOH) analysis with polymorphic microsatellite markers is a sensitive method which might reveal the presence of a tumour suppressor gene (TSG) and is often used to construct deletion maps. LOH at chromosome 6p has been described in many different tumour types and is one of the major mechanisms responsible for loss of HLA expression. The high frequency of LOH at especially 6p21.3 might also indicate that not

only the HLA genes but also one or more TSG present within the HLA-region are targeted by genomic loss. Loss of an entire chromosome, large hemizygous deletions and mitotic recombination will all result in LOH. The presence of contaminating normal cells is a major problem in this type of analysis as it may result in false-negative retention of markers leading to a “zebra pattern”. Although LOH analysis has been widely used in many different types of cancer in the search for TSG, few studies have addressed the mechanisms responsible for the observed allelic imbalance. In **chapter 3** we describe a detailed LOH analysis of 39 primary DLBCLs derived from the testis, CNS and lymph nodes using a large set of microsatellite markers on chromosome 6 with 12 being located in the HLA region. To elucidate the mechanisms responsible for LOH, we applied interphase FISH on 20 cases using PAC and cosmid probes specific for the HLA region in combination with a centromere 6 probe. In the majority of cases, LOH was caused by intrachromosomal deletions and not due to mitotic recombination or loss of an entire chromosome. Deletions as a result of a translocation, which is a recurring phenomenon in lymphomas, was observed in just one testicular lymphoma.

We applied an extended panel of HLA class I and II antibodies on tissue sections of much larger series of testicular (74), CNS (60) and nodal lymphomas (53) to confirm our previous results (**chapter 4**). To investigate whether loss of HLA expression was specific for testicular and CNS lymphomas we included extra-nodal DLBCL from two other sites, the stomach (22) and the skin (45). In total, we evaluated 254 cases. In line with our previous results, the testicular and CNS lymphomas showed complete loss of HLA-A expression in respectively 66% and 36% of the cases and complete loss of class II expression (HLA-DR/P/Q) in 53% and 40% of the cases. In comparison, the three other groups showed in 0-19% of the cases complete loss of HLA-A and in 0-5% of the cases loss of class II expression. As loss of HLA expression in tumours is thought to play a major role in immune evasion, we subsequently studied the number and nature of the tumour-infiltrating T cells in 43 testicular, 29 CNS, 14 stomach and 10 cutaneous lymphomas and compared this with a group of 70 nodal DLBCLs. High numbers of CD3<sup>+</sup> T cells were present in the testicular, the cutaneous and the nodal lymphomas. The testicular lymphomas showed the highest absolute number of immune cells positive for granzyme B<sup>+</sup> (GB), a marker for activated cytotoxic cells. Interestingly, the percentage of tumour-infiltrating T cells with an activated cytotoxic phenotype was also significantly higher in the testicular lymphomas (66%) compared to the CNS (44%), stomach (27%), cutaneous (24%) and nodal lymphomas (22%). To further characterize the cytotoxic T cell population, immunofluorescence double staining was performed on four CNS and five testicular DLBCLs. The majority of CTLs co-expressed CD3, a pan T cell marker and CD8 but we also detected small CTL populations

co-expressing CD3 and CD4. NK cells did not seem to play a major role in the host defense against these lymphomas as we saw in only one CNS lymphoma some scattered CD56<sup>+</sup> cells. We therefore hypothesized that compared to DLBCLs at various other sites, those DLBCLs arising at immune privileged sites are more immunogenic and are subject to selective pressure by the immune system. As a consequence, subclones without HLA expression experience major growth advantage as they have the probability to escape from CTL attack which might also be important as those lymphomas were not protected by expressing PI-9, a potent GB inhibitor, as has been reported in nodal DLBCLs.

Based on the striking differences in loss of HLA class II expression between the testicular and the nodal DLBCLs, we speculated that patients of both groups of lymphomas would show different frequencies of the different HLA-DR and DQ alleles. We therefore performed DNA-typing of the HLA-DRB1 and HLA-DQB1 genes in 50 patients with testicular DLBCL and 48 patients with nodal DLBCL and compared the distribution of the different alleles with a large cohort of healthy Dutch controls (**chapter 5**). When all patients were analysed as one group, they showed a positive association with HLA-DRB1\*15 and HLA-DRB1\*12 and a negative association with HLA-DRB1\*07. When the patient groups were analysed separately, the significant positive association with HLA-DRB1\*12 was seen in the patients with testicular DLBCL and the significant negative association with HLA-DRB1\*07 was seen in the patients with nodal DLBCL. Moreover, for many alleles different frequencies were seen when both groups were compared with each other, but due to the relatively small size of the patients groups no significance was reached after multiple corrections. Our results suggest that the different DR and DQ alleles play a role in susceptibility for DLBCL as well as the site of primary presentation. However, this is only the second report in the literature about the distribution of HLA-DR and DQ alleles in Caucasoid DLBCL patients and the first describing the frequencies in testicular and nodal DLBCL. So, to investigate whether specific HLA-DR/DQ genotypes are of real importance in lymphomagenesis, typing of larger groups of DLBCL should be performed and preferably, distinction should be made, between the different sites of primary presentation. In the final chapter (**chapter 6**) the role of HLA-DR in the development of DLBCL is being discussed. Initially during the immune response, signaling via HLA-DR is important for B cell proliferation and growth. Later on, HLA-DR signaling may result in apoptosis. Loss of HLA-DR expression may therefore be very important for tumour cell survival and HLA-DR could therefore be regarded as a tumour suppressor gene .

