Expression of human leukocyte antigens in diffuse large B cell lymphomas

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Chapter 3

Hemizygous deletions in the HLA-region account for loss of heterozygosity in the majority of diffuse large B-cell lymphomas of the testis and the central nervous system

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Abstract

Loss of heterozygosity (LOH) is a major mechanism for inactivation of tumour suppressor genes and has been observed in various solid tumours and lymphomas. The HLA-region is located at chromosome 6p21.3 and loss or alteration of this region may provide tumour cells with a mechanism to escape from the immune system. We previously identified small homozygous deletions within the HLA class II region in many of the diffuse large B-cell lymphoma (DLCL) of the central nervous system (CNS) and the testis. In the present study we focused on the mechanism leading to LOH in the HLA region. Twenty microsatellite markers of which 12 specific for HLA, were applied on 11 extranodal DLCL of the CNS and 28 of the testis. Additionally, fluorescent in situ hybridization (FISH) with seven HLA specific probes and a centromere 6 specific probe was performed on 20 cases to study the mechanism of LOH. In contrast to previously published data on spontaneously mutated lymphoblastoid cell lines, intra-chromosomal hemizygous deletion and not mitotic recombination was the major cause of LOH of the HLA region in these lymphomas. However, opposed to data in colorectal cancer, these deletions were rarely (one of nine cases) associated with an inter-chromosomal rearrangement such as a translocation.

Introduction

In many human neoplasms, multiple genetic events such as deletions, mutations, non-disjunction and mitotic recombination are required for the malignant transformation of cells. Loss of heterozygosity (LOH) analysis with polymorphic microsatellite markers is a sensitive molecular method to screen for allelic loss which might reveal the presence of a tumour suppressor gene and is frequently used for construction of deletion maps. LOH can be caused by point mutation, deletion or loss of a whole chromosome but also by mitotic recombination.

Many microsatellite markers have been identified within the human leukocyte antigen (HLA) region on chromosome 6p21.3 [1,2] which harbours approximately 130 genes and pseudogenes[3]. The HLA class I and class II molecules play an important role in initiating and regulating the T-cell mediated anti-tumour response [4]. Loss of HLA surface expression has been described in numerous human solid tumours, tumour cell lines as well as B-cell lymphomas [5-7] and this is thought to result in escape from cytotoxic T-cell attack.

LOH of chromosome 6p has been reported to be a common event in the etiology of many different neoplasms, indicating the presence of potential tumour suppressor genes in this region [8-16]. Several mechanisms explaining LOH at 6p have been described including large hemizygous deletions, mitotic recombination or loss of an entire chromosome with or without concomitant duplication of the other chromosome [17-20]. Moreover, we previously demonstrated small homozygous deletions of the HLA-DR and -DQ in the HLA class II region [7], suggesting that the genes are an important target of inactivation [21].

When interpreting data obtained by LOH analysis, allelic loss can not be distinguished from allelic imbalance (AI) caused by trisomy or other aneusomies. Moreover, the admixture of normal cells in a tumour is an obvious cause of error in LOH studies as the markers are differently affected by this admixture [22]. These factors often lead to the “zebra pattern” observed in many LOH studies [23]. To overcome these problems and to study the mechanisms causing LOH a combination of cytogenetics and/or FISH with LOH analysis is obligatory. Here we report on the detailed LOH analysis of 39 primary DLCL cases of the CNS and the testis using 20 markers on chromosome 6, of which 12 were located in the HLA-region. To elucidate the mechanism that caused LOH at 6p we applied interphase FISH on 20 cases, including three cases with no or minimal LOH, using seven HLA class I, II and III region specific probes and a centromere 6 probe. Applying FISH, we also investigated whether LOH could be the result of inter-chromosomal translocations or inversions accompanied by deletion of DNA, as recently found for colorectal cancer [24]. We show that

interstitial deletion without inter-chromosomal translocation is the most common mechanism accounting for the observed LOH in extranodal DLCL.

Materials and Methods

Tissue Samples

Thirty-nine diffuse large B-cell lymphomas according to the Revised European-American Lymphoma classification [25] were collected. The B-cell origin was confirmed by immunohistochemical staining for CD19, CD20, CD22 or CD79a. From these DLCL, 11 were of primary cerebral origin and 28 of primary testicular origin. Tissue blocks from these cases were retrieved from the tissue bank of the Pathology Department at the Leiden University Medical Center (LUMC, Leiden, The Netherlands), from Dr. L. Looijenga from the Josephine Nefkens Institute (Rotterdam, The Netherlands) or from the NHL Registry of the Comprehensive Cancer Center West in the Netherlands between 1981 and 1989.

Microdissection and DNA Extraction

DNA was extracted according to the protocol described by Isola et al. [26] with some adjustments. Paraffin-embedded tissue of the 39 cases was cut in 10 μm sections and haematoxylin and eosin-stained. Before the normal dehydration steps, the staining procedure was interrupted to use the slides for microdissection. To enrich for tumour cells, selected areas containing over 70% tumour cells were microdissected using a needle under direct light microscopic visualization. Normal control tissue was obtained using the same procedure. DNA was extracted by incubation for 72 hours at 56°C in 1 mL of isolation buffer (100 mM $NaCl_2$, 10 mM Tris-HCl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS). 30 μL aliquot of proteinase K (10μg/μL) was added and this step was repeated 24 and 48 hours later. Phenol-chloroform-isoamyl alcohol was used for DNA isolation, 1 mL of 100% ethanol, 20 μg/mL glycogen and 250 μL 7.5 M ammonium acetate for precipitation and the obtained pellet was dissolved in Tris-EDTA (10 mM Tris, 0.1 mM EDTA, pH 7.6). 1 μL was used as a template for PCR.

LOH Analysis

DNA from normal and tumour microdissected material from all 39 DLCL cases was analyzed for LOH by PCR amplification using 20 highly informative microsatellite markers (heterozygosity ranging from 56% to 100%). The primer sequences for the microsatellite markers have been described before [9,32] .

Standard PCR amplifications [10] were carried out in a 12 μL reaction volume containing 1 μL purified template DNA, 6pmol of each primer, 2 mM dNTPs, 0.1 mg/mL BSA, *Taq* polymerase buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatine, 0.1% Triton), 0.06 units Super*Taq* polymerase (Sphaero Q, HT Biotechnology, Cambridge, UK) and 1 μCi [α-^{32}P]-CTP (Amersham, Buckinghamshire, UK). Samples were denatured for 5 min and amplified for 33 cycles consisting of 1 min denaturation at 94°C, 2 min primer annealing at 55°C, 1 min elongation at 72°C followed by a final extension step of 6 min at 72°C. The amplification reactions were carried out in 96-wells plates (DYNAX DPC, Breda, The Netherlands) using a thermal cycler (MJ Research, Watertown, MA, USA). Radiolabelled products were denatured in formamide loading dye and analyzed on 6% polyacrylamide gels. Dried gels were autoradiographed at room temperature for 18-24 hours. The Molecular Dynamics Phosporimager 445SI (Molecular Dynamics, Sunnyvale, CA, USA) was used for quantification of the PCR products. An allelic imbalance factor was calculated by the quotient of the ratios of the peak heights from normal and tumour DNA. Each PCR reaction was performed at least twice. An imbalance factor ≥ 1.7 was considered LOH. An imbalance factor between 1,4 and 1,7 was also regarded as LOH if the adjacent markers showed unambiguous LOH but was depicted separately (Fig. 1).

Interphase FISH analysis on nuclei isolated from frozen material

In 16 cases, interphase FISH analysis was performed on nuclei isolated from snap-frozen tissue using seven HLA-region specific probes and a probe for centromere 6 [7] (Fig. 4A). The α-satellite centromeric 6-probe (D6Z1), biotin-16dUTP- or digoxenin-12-dUTP- labelled (Roche, Basel, Switzerland), was obtained from Vysis (Downers Grove, IL, USA). PAC 223H1 and PAC 238M10 were isolated from the RCPI-1 Human PAC Library of the Roswell Park Cancer Institute (obtained by Dr. J. Den Dunnen, Genome Technology Center, LUMC, Leiden) using respectively a TAP1 cDNA probe and a HLA-C probe. PACs 93N13 and 172K2 were directly obtained from the RCPI 1-library (GenBank Ac.nr: Z84489; Z84814). Cosmid 619pWE1 was kindly provided by Dr. H. Inoko (Tokai University School of Medicine, Kanagawa, Japan). Cosmid

c109K2118 derived from the ICRF flow-sorted chromosome 6 library was obtained from the Resource Center/Primary Database of the German Human Genome Project (Berlin, Germany) and cosmid M31A from the American Tissue Culture Center. Cosmid and PAC probes were labelled with digoxenin-12-dUTP or biotin-16dUTP (Roche, Basel, Switzerland) by standard nick translation. Hybridization and immunodetection were performed as previously described[27]. Slides were analyzed with a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany). Images were captured using a COHU 4910 series monochrome CCD camera (COHU, San Diego, CA) attached to the fluorescence microscope equipped with a PL Fluotar 100×, NA 1.30 to 0.60 objective and I3 and N2.1 filters (Leica) and Leica QFISH software (Leica Imaging Systems, Cambridge, UK). Images were processed with Paintshop Pro and Corel Draw 8.0. Ten tonsils of healthy individuals were used as controls. The cut-off level for homozygous and hemizygous deletions, was set at the average of the controls plus three times the SD (Table 1).

Interphase FISH on nuclei isolated from paraffin-embedded tissue

The protocol for interphase FISH on nuclei isolated from paraffin-embedded tissue was adapted from the protocol for hybridization on paraffin sections described by Haralambieva et al. [28]. Four testicular DLCL cases with only paraffin material available were analyzed. Two 50 μm sections of paraffin-embedded tissue were cut and transferred to 15ml tubes. The sections were dewaxed 3 times for 5 min in 3 ml xylol and rehydrated. 3ml Tris-EDTA (50 mM Tris, 2 mM EDTA, pH9) solution was added to the sections and the samples were pressure-cooked in a CentroClav steam-sterilizer (Type CV-EL; KELOMAT, Traun, Austria) for 10 min. The sections were afterwards washed 2 times in 2XSSC and incubated with RNAse A (100μg/ml) for 1 hour at 37°C in a water bath. After washing 2 times with 2XSSC the sections were pre-treated with 0,5% pepsin (w/v; Serva, Heidelberg, Germany) in distilled water at pH2 at 37°C for 30 min. The cells were then washed 2 times with PBS and filtered through a nylon filter (Verseidag-industrietextilen GMBH, Kempen, Germany). An appropriate number of cell nuclei was diluted in PBS, to which methanol: acetic acid (3:1) was added drop by drop during shaking. The suspension was applied on microscope slides as described for interphase FISH on frozen material[27]. The slides were air-dried and used for hybridization. The latter was performed as described

for interphase FISH on frozen material with the only difference being a denaturation step of 12 min at 80°C instead of 3 min. The same DNA probe combinations were used for the hybridizations as for nuclei isolated from frozen material (see above). Three tonsils of healthy individuals were used as controls and as the results were similar to the results obtained with the ten frozen tissue samples (see above) the same cut-off levels were used for determining the deletions.

Translocation detection

To detect potential translocations (or inversions) FISH analysis was performed in 12 cases. A biotin-16dUTP- and a digoxenin-12-dUTP-labeled probe flanking each homozygous deletion (T20, T29, C9, T28, T25, T27, T14 C1, T2, T16, T19, T18) and hemizygous deletion confined to the HLA region (C9, T28, C1, T2, T16, T19, T18) were co-hybridized on interphase nuclei. Hybridization and immunodetection were performed as described above. A translocation or inversion was detected by segregation of the two probes.

Results

Allelotyping analysis

We studied 39 DLCL cases for allelic imbalances at chromosome 6. Seventeen polymorphic microsatellite markers were localized at 6p including 12 markers within the HLA-region. Three markers were localized at 6q. The results were classified according to the extend of observed LOH in the CNS and the testicular DLCL cases from left to right (Fig. 1). The majority of the 28 testicular lymphomas and approximately half of the 11 CNS lymphomas showed extensive LOH at 6p, in particular in the HLA-region. LOH at 6q was equally frequent in both groups (50%) which is in concordance with the literature [29].

Three cases (T23, T13, T11) showed LOH at all informative markers at 6p while ten lymphomas (C1, T5, T15, T18, T14, T29, T20, T21, T30, T12) at nearly all markers mostly including the telomeric end, indicating the presence of a terminal deletion of 6p or mitotic recombination. Eight lymphomas (C9, C2, T2, T16, T24, T25, T7, T27) showed LOH at several markers at 6p confined to the HLA-region.

Four tumours (C5, C8, T2, T28) showed complex patterns with small regions of LOH interrupted by conserved domains. Besides, case T16 showed an imbalance factor between 1,4 and 1,7 at markers TY2A, BAT2 and C47, LOH at five other markers and retention of MICA. An allelic imbalance factor between 1,4 and 1,7 was considered as LOH if the flanking markers showed unambiguous LOH (an imbalance factor higher than 1,7), and as retention of heterozygosity if the flanking markers did not show any imbalance. Four other tumours (C7, T19, T24, T13) showed similar "zebra patterns" with alternating regions of LOH and regions with an imbalance factor between 1,4 and 1,7. In cases T16 and T19, the zebra pattern might also be due to the presence of relatively many normal cells in the specimen, resulting in generally low imbalance factors. In other cases individual markers were difficult to interpret because of strong shadow bands (case T29 at marker D6273, T13 at markers TNFa and C125). Case T26 showed a difficult to interpret pattern with retention of heterozygosity and interspersed markers with an imbalance factor between 1,4 and 1,7.

Four tumours (T6, T4, T3, T9) showed LOH at a few markers within the HLA-region and some additional markers at the telomeric and/or centromeric end of 6p.

Four tumours (C10, C3, C4, T22) showed LOH at only one marker and four tumours (C11, C6, T8, T10) at none of the markers located within the HLA-region. Three of these cases (C11, C6, T8) did not show LOH at any of the markers at chromosome 6. The highest percentage of LOH (19/25) was seen at marker TNFa in the testicular lymphomas and at marker D6S1666 (8/11) in the CNS lymphomas. Representative cases are depicted on Fig. 2 showing loss of the high molecular weight allele or the low molecular weight allele. In one case (T13) strong shadowbands were observed indicating the difficulties often encountered when studying LOH. Besides the three cases with retention of heterozygosity at marker D6S1666 (T18, T20, T25) we described previously [7], two more cases also showed retention at C47 (T14, T29) indicating an extended homozygous deletion.

Two separate events must have occurred at 6p and 6q, as in nearly all cases showing LOH at both arms the LOH pattern was interrupted by retention of markers D61017and D6S271 (except for T29, T20, T11) centromeric of the HLA-region.

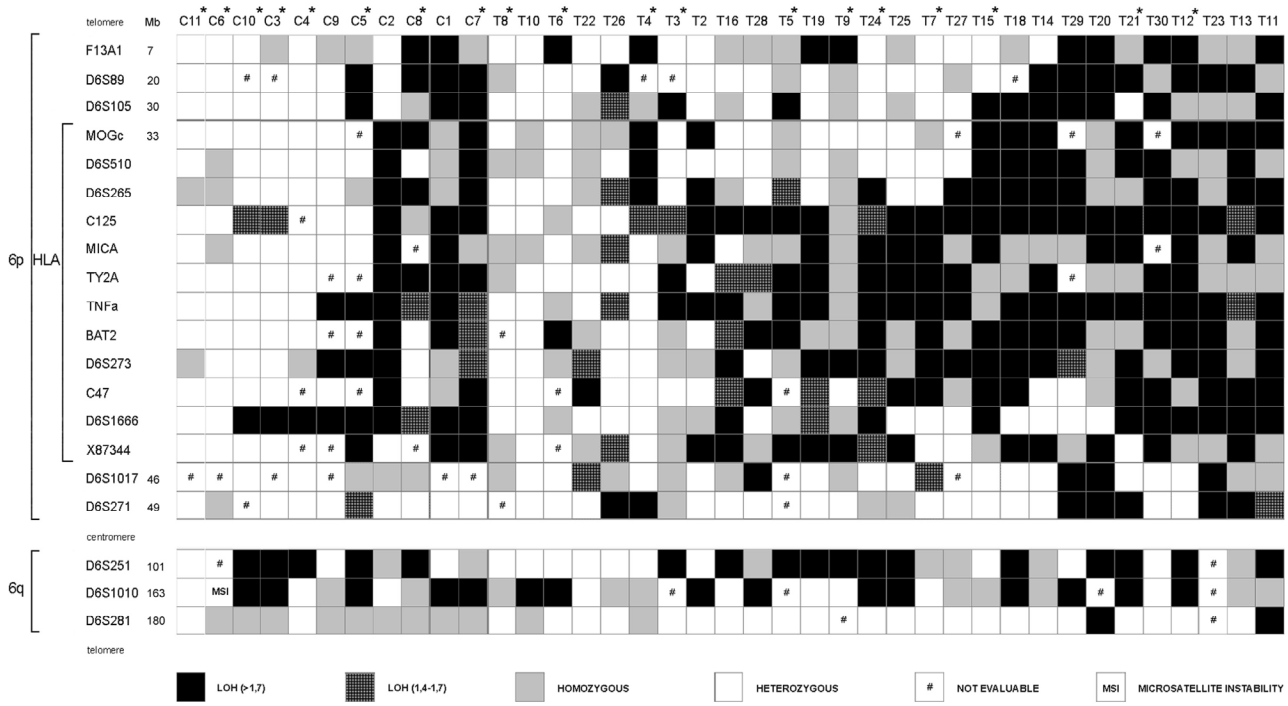

Figure 1. 11 CNS (C) and 28 testicular DLCL (T) were studied by microsatellite analysis using 20 polymorphic markers at chromosome 6. The approximate location of the markers centromeric and telomeric of the HLA-region is shown (http://bioinformatics.weizmann.ac.il/udb_21a/). Individual cases are classified according to the extent of LOH at the HLA-region from left to right per tumour type. Cases with no available material for FISH analysis are marked by an asterisk.

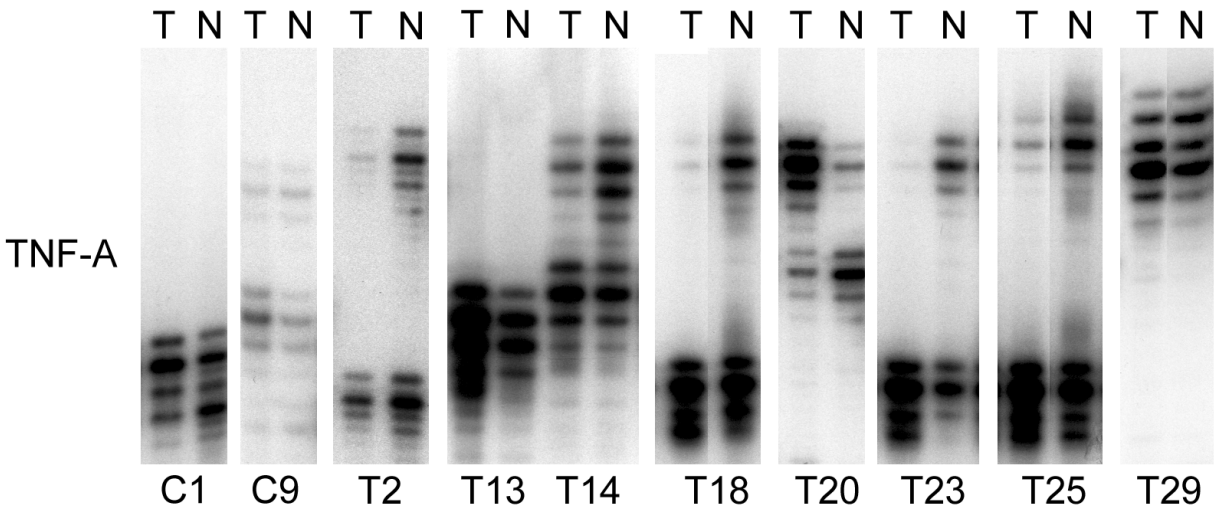

Figure 2. Autoradiograms demonstrating LOH at marker TNFa in ten representative CNS and testicular DLCL cases. Loss of the H allele was detected in cases C9, T2, T14, T18, T23, T25 and T29. Loss of the L allele was detected in cases C1, T13, and T20. Strong shadowbands of the H allele were observed in case T13.

Interphase FISH results

Interphase FISH analysis was performed on nuclei isolated from frozen material or paraffin sections of 20 of the lymphomas described above. In all cases, seven HLA-region specific clones including two for the HLA class I region, one for the HLA class

III region and four for the class II region, in combination with a probe for centromere 6 were applied. Absence of both HLA-region specific signals was scored as a homozygous deletion and absence of one signal as a hemizygous deletion (see Fig. 4 for representative cases and Fig.3A for probes used).

Large (> 4 Mb) hemizygous deletions and small homozygous deletions comprising the HLA class II region were detected in the majority of cases (see Results section below). The cut-off level for these deletions was calculated for each PAC or cosmid clone using data from ten healthy controls (Table 1). In three cases (T23, T2 and T18) we found trisomy 6 in respectively 70%, 10% and 20% of the tumour cells and in case T25 we found tetrasomy in approximately 20% of the tumour cells.

Probe	Location	Cut-off level homozygous deletion*	Cut-off level hemizygous deletion*
C109K2118	HLA-A	1%	15%
238M10	HLA-B, -C	11%	18%
M31A	TNFα	2%	21%
172K2	DRA	7%	15%
93N13	DRB; DQ	3%	14%
223H1	TAP	6%	10%
619pWE15	Centromeric part HLA class II	2%	9%

Table 1. Combined LOH and interphase FISH analyses
Cut-off levels for determining homozygous and hemizygous deletions for the PAC and cosmid clones used for interphase FISH.* determined as the average percentage of nuclei of ten tonsil controls showing loss of one or two probe specific signals as compared to the number of centromere 6 signals. Cut-off levels are represented as the average plus three times the SD.

Combined LOH and Interphase FISH Analysis

The LOH and FISH results of 13 representative cases are depicted in Fig. 3B-D and grouped in several different categories with respect to the observed aberrations. Of the remaining seven cases, tumour T27 showed a pattern similar to T25 and tumour T11 similar to T13. Cases T16 and T19 resembled the aberrations seen in T25 but at lower percentages, probably due to higher numbers of infiltrating normal cells. These cases are represented between brackets in Fig. 3. Cases T10, T22 and T26 with no or sporadic LOH did not show

any aberrations with FISH (data not shown). As previously described [7], the retention of heterozygosity for markers D6S1666 and C47 in several testicular lymphomas (see above) was due to small homozygous deletions in the tumour cells with PCR amplification of the alleles of the contaminating normal cells [30].

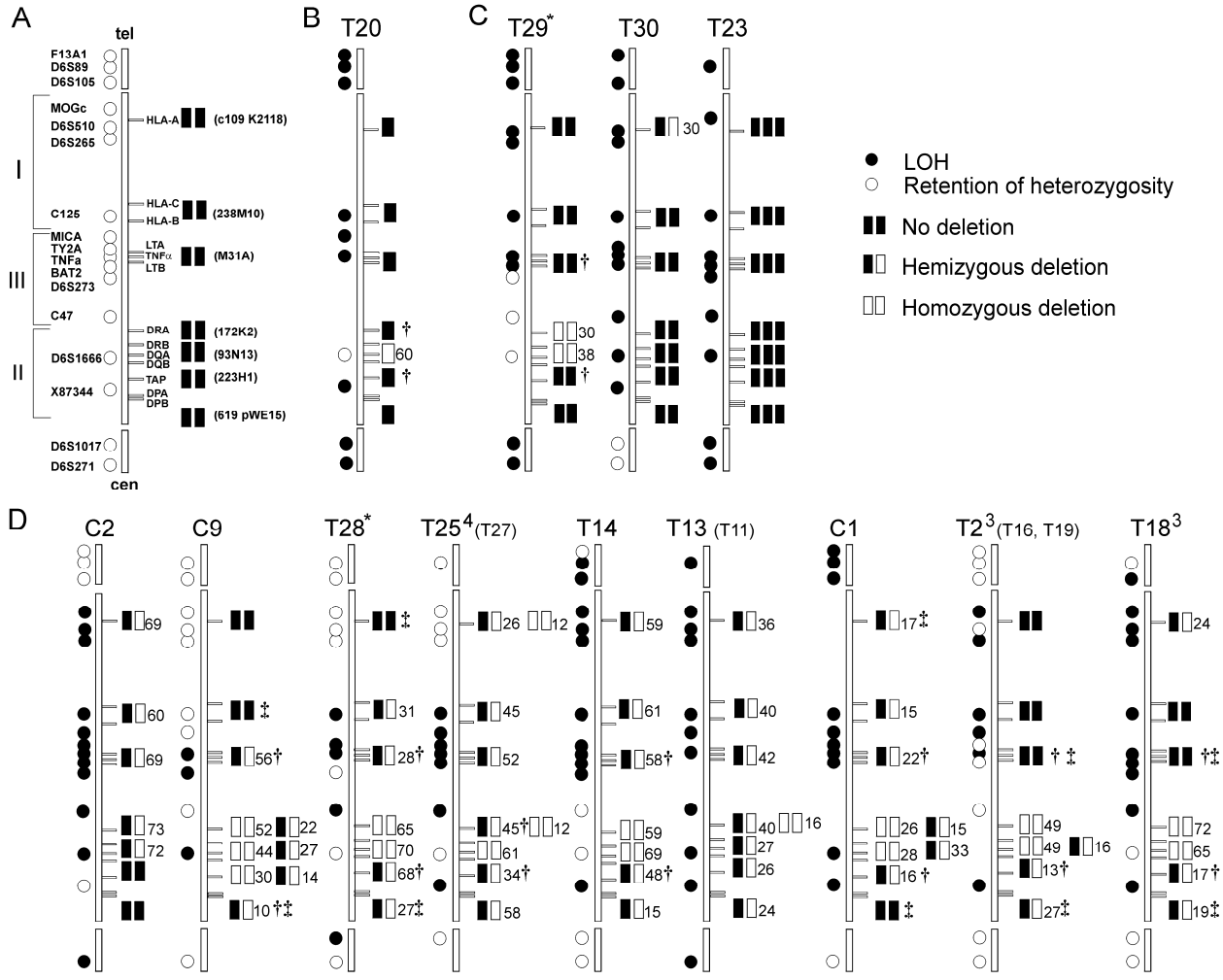

Figure 3. Summary of the LOH and interphase FISH results of 17 representative DLCL cases. Three cases (T10, T22, T26) showed no or minimal LOH and no abnormalities by FISH and are not shown.
(A) Schematic representation of the HLA-region on chromosome 6p. On the left side are illustrated the 12 microsatellite markers in the HLA class I, II and III region and the five markers situated at the telomeric and centromeric sides. On the right side are depicted the seven PAC and cosmid clones used for interphase FISH and the corresponding genes.
(B-D) For each individual tumour, the LOH results are represented at the left. Only informative markers are shown. The FISH results with the percentage of aberrant nuclei are shown on the right. The number of rectangles represents the number of centromere 6 signals in the majority of nuclei. Heterogeneity within the tumour samples is indicated: "3", trisomy 6 (T2: 10%; T18: 20%); "4", tetrasomy 6 (T25: 20%). "*" lymphomas studied by FISH on paraffin-embedded material. "†" and "‡" probes used for translocation studies of respectively homo- and hemizygous deletions in individual cases.
(B) Lymphoma case with monosomy 6
(C) Lymphoma cases with mitotic recombination (T29, T30) and non-disjunction (T23)
(D) Lymphoma cases with large hemizygous deletions, either or not in combination with mitotic recombination

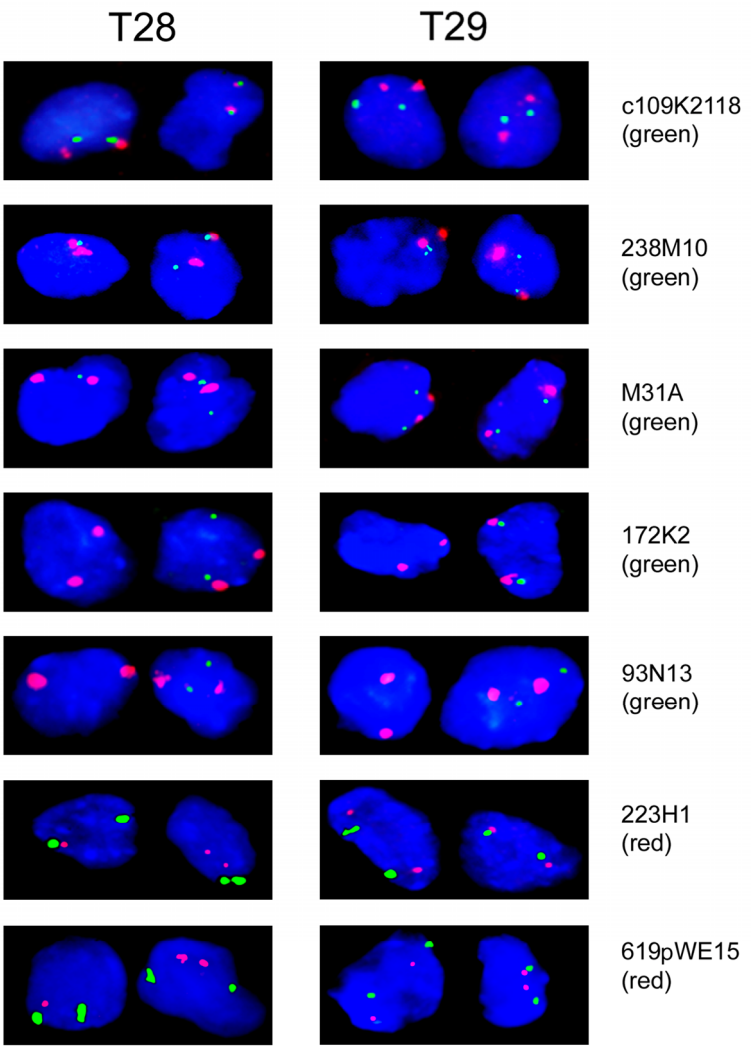

Figure 4. FISH analysis of interphase nuclei isolated from paraffin-embedded material of two representative lymphoma cases. The seven PAC and cosmid clones (shown on the right) were combined with centromere 6 in all hybridizations (see also Fig. 3A). A composite panel of a normal and an aberrant nucleus is shown for each clone.

Lymphoma case with monosomy 6 (Fig. 3B)

In case T20 the presence of only one signal for centromere 6 and the LOH pattern pointed to a monosomy 6. The retention of heterozygosity at marker D6S1666 was due to a homozygous deletion of approximately 500kb including PAC93N13, which contains this microsatellite marker.

Lymphoma cases with mitotic recombination or non-disjunction (Fig. 3C)

In two tumours (T29, T30) with extensive LOH, two HLA-region specific signals were detected by FISH. In case T29 the retention of heterozygosity at markers D6S1666 and C47 was in line with the observed homozygous deletion in the HLA class II and class III region. Therefore, it is plausible that initially a relatively small intra-chromosomal deletion of the class II and III region occurred at one chromosome followed by homogenization due to mitotic recombination. The LOH pattern in case T30 is compatible with mitotic

recombination and an additional hemizygous deletion of the region covered by the probe for HLA-A (detected by FISH in 30% of the nuclei).

Tumour T23 showed three signals for all probes including centromere 6 and LOH at all markers at 6p, mostly with complete loss of one of the alleles. This probably resulted from mitotic recombination of at least 6p and subsequent non-disjunction leading to the presence of three copies of the same chromosome 6 in each nucleus. However, as the LOH pattern of the markers located on 6q could not be assessed we can not exclude the possibility of non-disjunction leading to chromosome loss followed by triplication of the remaining copy of chromosome 6.

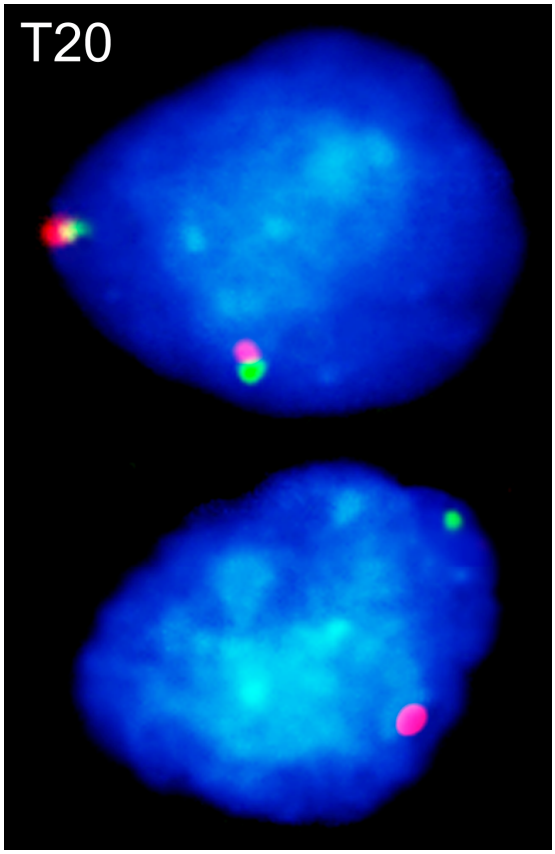

Figure 5. Translocation detection using interphase FISH in case T20. A composite panel of one normal and one tumour nucleus is shown. PAC172K2 (green) and PAC223H1 (red) were co-hybridized. Segregation of the two signals indicates a translocation of the telomeric part of 6p.

Lymphoma cases with large hemizygous deletions

All remaining 13 cases showed hemizygous deletions with a minimal length of 1,5 Mb (Fig. 3D) In cases C2 and T13 the observed hemizygous deletion was compatible with the LOH pattern. In case T13 a minor but significant subset of nuclei also showed a homozygous deletion of probe 172K2, indicating a second hit in a sub-population of the tumour cells.

Also in T28, T25, T14 and T18, at least two hits had occurred as a homozygous deletion of the HLA class II region was seen in addition to the hemizygous deletions.

In case C9 and C1 the LOH at D6S1666 could be explained by intra-tumour heterogeneity as next to tumour cells with a homozygous deletion of this region a sub-population with a hemizygous deletion (27-33%) was observed.
In case T28, a relatively low percentage of nuclei with a hemizygous deletion (30%) resulted in LOH at C47, C125 and the TNF genes while retention was seen at marker D6S273. Probably, LOH at this marker in the mixed cell population was more difficult to detect than for the adjacent markers.
In case T25, a small tetraploid population with the same aberrations as the diploid cells was present. Moreover, within the HLA-A region a hemizygous deletion was found in 25% of the nuclei but this did not result in LOH.
Case T2 showed a typical complex "zebra pattern". Retention of heterozygosity at marker TY2A in the class III region might indicate the presence of a small homozygous deletion but this could not be confirmed by interphase FISH (data not shown). In this tumour, LOH at the class III and I region was probably due to mitotic recombination as no deletions were detected by FISH. No explanation was found for the "zebra pattern" in the telomeric part of the class I region as marker D6S510 showed a strong signal with both alleles being 6bp apart and no overlapping shadowbands (data not shown).
In case T18, a small interstitial deletion in the class II region was probably followed by recombination of at least 5Mb including the whole HLA-region. In 20% of the tumour cells we detected three copies of chromosome 6 indicative of non-disjunction. In a small proportion of the cells also a hemizygous deletion occurred in the HLA-A region and the centromeric part of the class II region.

Translocation detection

In all cases with a homozygous deletion or a hemizygous deletion restricted to the HLA region (T20, T29, C9, T28, T25, T27, T14, C1, T2, T16, T19, T18) (see figure 4), we co-hybridized differently labelled probes located on the centromeric and telomeric part of the deletion to determine whether the observed deletions were associated with translocations or inversions. For each case an optimal probe set was used. In case T20, segregation of the PAC172K2 and PAC223H1 signals was seen, indicating a translocation or inversion of the telomeric part of 6p (Fig. 5). In all other cases co-localization was observed.

Discussion

Tumour development is a complex multistage process and numerous genetic alterations including point mutations, deletions and numerical chromosomal aberrations are found in neoplastic cells of different tumour types. Many of these mutations are thought to be a consequence of acquired genetic instability during tumour development and progression, and often involve multiple chromosomes and genes including oncogenes and tumour suppressor genes.

In both normal and neoplastic somatic cells, spontaneous mutations occur frequently and this may lead to heterozygosity or on the opposite, if the affected genes already harbour germline or somatic mutations, loss of heterozygosity. At a heterozygous locus, LOH may result from a locus restricted event, such as point mutation, small intragenic deletion [31] or gene conversion, or from a multilocus chromosomal event such as a large deletion, mitotic recombination or chromosome loss with or without reduplication [32-35].

LOH analysis in tumours is frequently used as an indicator of genetic loss but the results are sometimes difficult to interpret and prone to misjudgement. Studying the possible effects of oxidative damage on human cell lines, [36] observed complex discontinuous LOH patterns. Furthermore, the contamination of tumour samples with varying amounts of normal cells has been shown to lead to complex LOH patterns of alternating regions with LOH and regions with retention of heterozygosity [22]. Such "zebra patterns" might be due to artificial retention of heterozygosity as different markers are affected to a different extend. Often the signal of the low molecular weight allele (L) is stronger than the signal of the high molecular weight allele (H) of the same locus [23,37] (see Fig. 2). Moreover, shadow bands from the H allele may increase the strength of the L allele signal. Liu et al. [23] showed that the detection of loss of the H allele is more sensitive than loss of the L allele in samples that contain normal cells. Based on these considerations, they suggested that about 50% of the L allele deletions might be missed by conventional LOH analysis.

In the present study of 39 lymphomas we observed very high frequencies of LOH at chromosome 6p, especially within the HLA-region (Fig. 1). Some LOH patterns were difficult to interpret despite the close proximity of the microsatellite markers used, with several tumours showing a complex "zebra pattern" of evident LOH alternated with regions with an imbalance factor of 1,4 to 1,7. This was in some cases due to a relatively high percentage of contaminating normal cells and in others to the presence of strong shadow bands of the H allele.

The HLA-region at chromosome 6p21.3 harbours the genes encoding for the HLA molecules, which present antigens to T cells, and numerous other genes involved in the immune regulation. This region showed high percentages of LOH in various solid tumours as well as

haematological malignancies [6,10,14,38], suggesting that loss of immune recognition is a common mechanism resulting in immune escape.

So far, few studies have been published investigating the mechanisms responsible for LOH of the HLA-region in the different tumour types affected. In spontaneously mutated lymphoblastoid cell lines, selected for loss of HLA-A2 expression, somatic recombination and loss of chromosome 6 with duplication of the remaining chromosome were found to be the most important mechanisms causing LOH [39]. In contrast, using a restricted set of microsatellite markers spanning chromosome 6, loss of one entire chromosome 6 was suggested to be more frequent than somatic recombination in melanomas, colonic and laryngeal tumours with LOH [18]. However, in the latter study no FISH analysis was performed to confirm this suggestion or to explore other possibilities as for example large deletions or mitotic recombination.

Recently, applying metaphase FISH, Thiagalingam et al. [24] showed that in colon carcinoma, LOH is more frequently associated with inter-chromosomal recombinations and deletions in combination with DNA double strand breaks than previously thought. Interestingly, in the presently investigated lymphomas, we confirmed that deletions and not chromosome loss or mitotic recombination, are the major cause of LOH. In contrast to Thiagalingam et al.[24], a translocation contiguous to the deletion was solely detected in one lymphoma, indicating that this is not a common event in DLCL. Of note, our method differed since we used interphase FISH with probes immediately flanking the deletion, whereas they used chromosome paint probes on metaphase preparations, a method that also detects more distant translocation breakpoints.

We conclude that sole use of LOH analysis is not sufficient and complementary FISH analysis is imperative to obtain insight in the mechanisms causing LOH in human malignancies.

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