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Allelic Losses on Chromosome 6q25 in Hodgkin and Reed Sternberg Cells[1]

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ABSTRACT

We established a molecular cytogenetic approach to identify consistent genetic aberrations in classical Hodgkin lymphoma. Single laser-micromanipulated Hodgkin and Reed Sternberg (H-RS) cells and the respective germ line tissue were PCR-amplified using highly polymorphic microsatellite probes. Loss of heterozygosity and genomic imbalances of the fluorochrome-labeled microsatellites were determined by fragment length analysis. Eleven cases of in classical Hodgkin lymphoma (cHL) were initially screened with 21 microsatellite markers scattered over the entire genome. Loss of heterozygosity was detected in >40% of informative loci in most cases indicating a deletion of a substantial part of the genome of H-RS cells. Allelic losses and imbalances on chromosome 6q were detected in most of these cases. A deletion mapping of 6q was performed in 16 cases of cHL. This detailed analysis of 6q led to the identification of a 3.3-Mbp region around *D6S311* flanked by *D6S978* and *D6S1564* that was altered in 11 of 14 cases of cHL analyzed. In conclusion, allelotyping of single H-RS cells revealed monoallelic chromosomal deletions and genomic imbalances on 6q that might affect genes critically involved in the pathogenesis of H-RS cells.

INTRODUCTION

H-RS[3] cells represent the malignant cells in classical Hodgkin lymphoma (cHL). These large multinuclear cells are characterized by their scarcity in the affected tissue. It was, therefore, only recently that single-cell studies disclosed the clonal germinal center B-cell derivation of H-RS cells in most instances (1–3). Nevertheless, H-RS cells lack most of B-cell specific phenotypic and molecular markers (4–6). One of the characteristic features of H-RS cells is the constitutive expression of NFκB (7), which might explain apoptosis resistance of these cells. In a portion of cHL, NFκB expression may result from the expression of the EBV encoded latent membrane protein-1 (LMP-1) or from deleterious mutations of the NFκB repressor IκBα (8–10). Mutations of genes involved in the pathogenesis of other B-cell lymphomas are rare or absent (11–14) in H-RS cells.

H-RS cells display chromosomal instability as reflected by recurrent cytogenetic aberrations, but not microsatellite instability (15, 16). Despite the presence of grossly abnormal karyotypes in H-RS cells, no specific chromosomal aberration has been identified thus far in these cells because classic cytogenetic analyses are hampered by the scarcity and the low mitotic index of the H-RS cells (17–19). Therefore, novel approaches like FISH or the combination of FISH and immunophenotyping have been applied to cHL. Results from these studies indicated (clonal) numerical or structural chromosomal aberrations in all cases of cHL (20, 21). More recently, H-RS cells were characterized by molecular cytogenetic methods like comparative genomic hybridization (22- 24) or LOH (15, 25, 26). These analyses indicated to the involvement of distinct genes in the pathogenesis of cHL (27, 28). Here we describe a high frequency of chromosomal deletions and imbalances in H-RS cells on 6q25 in 11 of 14 informative cases that might allow the identification of a novel chromosomal region possibly harboring tumor suppressor genes.

MATERIALS AND METHODS

Pathological Specimens. Pathologic specimens were classified according to the WHO classification (29). Characteristics of these cases are given in Table 1. From each sample, 5×10^5 cells were cytospun on a slide, air dried, and kryopreserved at −20°C. The corresponding H-RS cell-affected lymph nodes were kryopreserved in parallel. Peripheral blood samples were obtained at different time points. Cytospins were thawed at room temperature and stained with a monoclonal mouse antihuman CD30 antibody (clone Ber-H2; DAKO, Hamburg, Germany) and a secondary biotinylated rabbit antimouse antibody (clone E413, DAKO). Antibody reactions were detected with avidin-biotin-coupled alkaline phosphatase (DAKO) and FastRed as chromogen (DAKO). The EBV-negative H-RS cell line L1236 was established at our institution (30).

DNA Extraction. High-molecular weight germ line DNA was extracted from H-RS cell-affected lymph nodes or from peripheral blood mononuclear cells of the respective patient according to standard procedures. Tumor DNA was obtained from pooled H-RS cells after micromanipulation without DNA extraction. Single multinuclear CD30 positive H-RS cells were isolated using a noncontact PALM Laser MicroBeam System (Bernried, Germany). Multiple cells were collected in a microfuge cap and transferred to PCR tubes containing PCR buffer (Promega) as described previously (1).

PCR. Multiplex-PCR was carried out for 10 pooled H-RS cells from primary cases. The first round amplification was performed in a 25-μl reaction volume containing 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and a mixture of 21 primer pairs for screening experiments or a mixture of 7 primer pairs for mapping experiments (25 pmol of each). Microsatellites were chosen as suggested recently (31). Oligonucleotide sequences were derived from the Genome Database[4] and slightly modified. Internal nested oligonucleotides were designed using the respective DNA sequence and OLIGO software.[5] Physical and cytogenetic positions of microsatellites are given in Table 2. Forty cycles of denaturation (95°C for 30 min), annealing (57°C for 30 min), and elongation (72°C for 60 min) were performed in a personal cycler (Biometra, Göttingen, Germany) after adding 1 unit of *Taq* polymerase (Promega, Germany) during the first denaturation step. One microliter of the first PCR was reamplified in two separate reactions for each microsatellite as described before using 5′-labeled fluorescent oligonucleotides (6-carboxyfluorescein or hexachloro-6-carboxyfluorescein).

LOH Analysis. An aliquot of the PCR amplificate was mixed with a 6-carboxy-X-rhodamine-labeled size standard and analyzed for fragment length on a 377 DNA sequencer (Applied Biosystems/Perkin-Elmer, Foster City, CA). GeneScan software was used for the analysis of microsatellites. Loci were scored informative if the germ line was heterozygous. Loci were scored not informative if the germ line was homozygous or if no PCR product was reproducibly obtained from one of the sources. Allelic imbalance was scored if the height ratio of one of the tumor alleles compared with the height ratio of the germ-line alleles was <60% or >140% (31). LOH was scored only if one allele was completely absent.

RESULTS

H-RS Cells Frequently Display Allelic Losses (LOH). To avoid PCR amplification of incomplete chromosomal material, we used

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[3] The abbreviations used are: H-RS, Hodgkin and Reed Sternberg; FISH, fluorescence *in situ* hybridization; cHL, classical Hodgkin lymphoma; LOH, loss of Heterozygosity.

[4] Internet address: www.gdb.org.

[5] Internet address: www.oligo.net.

Table 1 *Characteristics of cHL cases of 15 individuals and the H-RS cell line L1236*

	Presentation	Subtype	CD15/CD30	EBV[a]	Stage
Case (identification no.)					
1404	First diagnosis	NS[b]	+/+	−	IIB
1476	First diagnosis	NS	+/+	−	IIIB
1622	First diagnosis	NS	+/+	−	IIA
1361[c]	First diagnosis	NS	+/+	−	IVB
1858[c]	First diagnosis	NS	+/+	+	IIA
1409	First diagnosis	MC	+/+	−	NA
1414	First diagnosis	MC	NA/+	+	IIA
1739	First diagnosis	MC	+/+	−	IIB
1494[c]	1st relapse	NS	+/+	−	IIA
1507	1st relapse	NS	+/+	+	IIIB
1454[c]	1st relapse	MC	+/+	−	IIA
1573[c]	2nd relapse	NS	+/+	−	IIA
1412	3rd relapse	NS	−/+	NA	IVB
1584	3rd relapse	LD	+/+	+	IA
1566	4th relapse	NS	NA/+	(+)	IIIA
Cell line					
L1236	3rd relapse	MC	+/+	−	IVB

[a] EBV status was tested by performing immunohistochemistry for LMP1.
[b] NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted; LR, lymphocyte rich classical HL; NA, not analyzed.
[c] Cases, that were analyzed exclusively with microsatellite markers located on 6q.

Table 2 *Physical position and cytogenetic localization of microsatellite markers*

(*A*) 15 screening markers on 9 different chromosomes and (*B*) 13 mapping markers on chromosome 6q.

Locus	Physical position in kbp (GeneMap 99)[a]	Cytogenetic localization (ensembl database)[b]
A		
D1S237	223274	1q32.1
D1S2827	224700	1q32.1
D2S391	29867	2p23.1
D3S1300	60350	3p21.31
D3S1530	136178	3q21.1
D3S1580	194164	3q26.32
D6S470	10344	6p24.2
D7S486	113538	7q31.1
D11S1345	133086	11q24.1
D11S1356	128111	11q23.3
D12S89	16812	12p12.3
D12S98	17421	12p12.3
D13S153	46413	13q14.2
D18S1156	26866	18q11.2
D18S35	53419	18q21.1
B		
D6S1709	109190	6q16.3
D6S1592	113610	6q21
D6S977	146600	6q24.1
D6S308	151575	6q24.2
D6S310	154606	6q24.3
D6S978	157540	6q25.1
D6S311	159554	6q25.2
D6S1564	160849	6q25.2
D6S440	163959	6q25.3
D6S1599	171725	6q26
D6S441	172328	6q26
D6S1590	183219	6q27
D6S281	184084	6q27

[a] www.ncbi.nlm.nihogov/genemap99/
[b] www.ensembl.org

CD30-stained cytospins instead of lymph node sections for micromanipulation of H-RS cells (Fig. 1). In a first screening experiment, 10 cases of cHL and, in addition, the H-RS cell line L1236 were analyzed for LOH. Analysis of L1236 cells was possible because germ line tissue from the patient from whom the cell line was derived has been available for PCR analysis. Twenty-one microsatellite markers located on 9 different chromosomes were PCR-amplified from pooled H-RS cells, and the respective germ-line DNA. In five cases, H-RS cells were compared with both germ-line DNA from the H-RS cell-affected whole lymph node and DNA extracted from peripheral mononuclear cells from the respective patient. Because the microsatellite pattern did not differ between DNA extracted from the two sources (data not shown), we further on compared H-RS cell DNA solely with DNA obtained from lymph nodes.

In 9 of these 11 cases, allelic losses were detected in at least 40% at informative loci, indicating a high degree of allelic losses in H-RS cells (see an example in Fig. 2). In the remaining two cases, LOH was detected in 0% and 13%, respectively (Table 3). The overall rate of complete allelic losses was 46%. Allelic imbalances were detected in 5% of analyzed microsatellite markers. In the 10 primary cases of cHL, complete allelic losses were scattered over nearly all chromosomes with a range from 0% (*D18S1156*) to 78% (*D6S311*), whereas H-RS cells of the cell line L1236 showed LOH on the long arm of chromosome 6 (6q). The microsatellite marker *D6S311* on 6q25 displayed LOH in seven of nine informative cases (78%), including the cell line L1236. Deletions on 6q were confirmed by a second marker (*D6S441*) located telomeric of *D6S311*, which showed allelic losses in 60% of informative cases. Other markers showing frequently allelic losses include *D2S391* (57%) and *D3S1580* (67%) on chromosomes 2p23 and 3q26, respectively.

The methodological validity of this single-cell approach was tested by repeating several second-round PCRs for markers located on 6q. Thirty-three of 36 markers that could be amplified successfully showed an identical allelic pattern (92%). To show that both the seminested PCR strategy and the micromanipulation strategy represented robust methods, a second control experiment was performed. Single H-RS cells of case no. 1507 were micromanipulated in a second effort independently of the first experiment. Ninety-three percent of fragment-length analyses were identical.

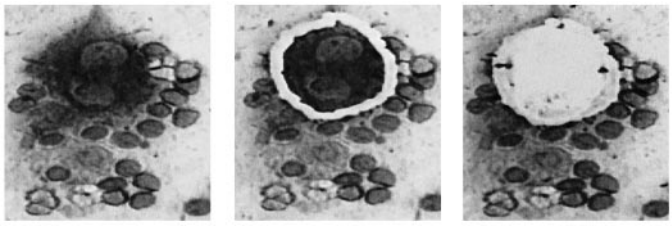


Fig. 1. Micromanipulation of single multinuclear CD30-positive H-RS cells from cytospins using a P.A.L.M. microscope (Bernried, Germany). Shown is a typical sequence during micromanipulation: identification of the H-RS cell, laser-ablation of bystanding cells, and isolation of the H-RS cell by a nontouch laser beam. The cell was transferred to a microfuge tube and used for multiplex PCR as described in "Material and Methods" (× 1000).

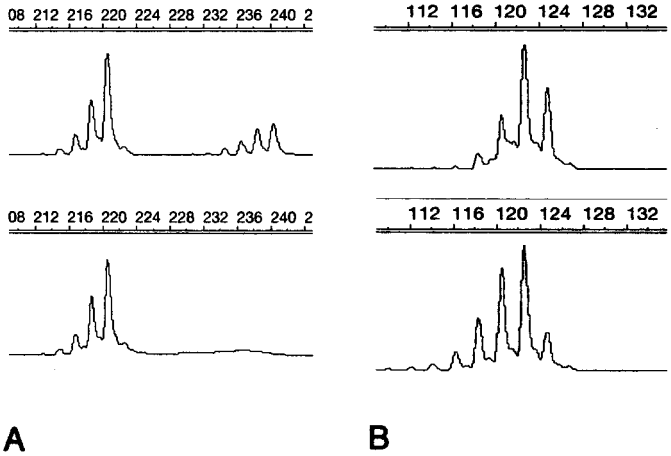


Fig. 2. GeneScan analysis of microsatellite markers for detection of LOH. Alleles were labelled with a fluorescent dye during PCR and analyzed for allelic heterozygosity. *A*, example of a complete LOH in H-RS cells. Allelic pattern of *D6S311* in a lymph node (identification no. 1507; *above*) and in pooled H-RS cells of that case (*below*) indicates a complete LOH for the 240-bp allele in the tumor cells. *B*, example of an genomic imbalance in H-RS cells. Comparison of *D6S1599* alleles between the lymph node (identification no. 1494; *above*) and the respective micromanipulated pooled H-RS cells (*below*).

Table 3 *LOH analysis of pooled H-RS cells of 10 cHL cases and the H-RS cell line L1236*

Results of a seminested multiplex PCR using 21 microsatellite markers are shown. *Black filled boxes*, complete LOH; *black filled boxes with open circles*, imbalances; *gray filled boxes*, no LOH; *blank boxes*, no PCR product. Identification numbers of cases correspond to Table 1. LOH is calculated on the basis of informative markers. *ni*, not informative.

Case / Marker	1409	1414	1476	1566	1584	1507	1622	1739	L1236	1404	1412	LOH (%)
D1S237	ni			●	●			●				22
D1S2827								ni	ni	ni		33
D2S391			ni		ni		ni	ni	●			57
D3S1300					ni			ni				29
D3S1530	ni				ni			ni				38
D3S1580				●					ni			67
D6S470	ni		ni	ni	ni							57
D6S1709	ni				ni						ni	67
D6S1592	●	ni					ni		ni		ni	29
D6S310			ni		ni							38
D6S308		ni		ni	ni	ni						50
D6S311										ni		78
D6S441			ni						ni	●	ni	60
D7S486	ni					ni	ni	●		ni		0
D11S1345	ni										ni	38
D11S1356	ni			ni								20
D12S89		ni										50
D12S98	ni		ni		ni							75
D13S153						ni						56
D18S1156	ni	ni	ni		ni	ni	ni	ni	ni		ni	0
D18S35									ni			44
LOH (%)	33	67	56	47	50	67	100	79	33	0	13	

Recurrent LOH on 6q25 in H-RS Cells. A detailed deletion mapping of 6q was performed next using seven additional microsatellite markers around *D6S311* and *D6S441*. Markers *D6S977*, *D6S978*, *D6S1564*, and *D6S440* are located in the vicinity of *D6S311*, whereas the marker *D6S1599* is close to *D6S441*. *D6S1590* and *D6S281* are located telomeric of *D6S441* on 6q27. Using these seven 6q markers and the six 6q markers of the screening experiment, we analyzed five additional cHL cases for LOH on 6q. Thus, a total of 16 cHL cases (including the 10 cHL cases and the cell line L1236) were tested for LOH on 6q with 13 microsatellite markers.

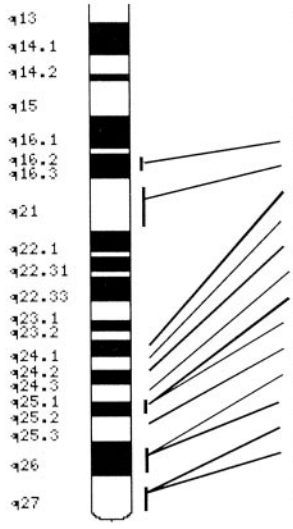
Allelic losses of *D6S311* on 6q25 detected in the screening experiment were found in two more cases of cHD out of five additional informative cases. The three remaining informative cases showed genetic imbalances at *D6S311*, thus not ruling out the possibility of incomplete allelic losses of this marker. In summary, 11 of 14 (79%) analyzed cases displayed genetic aberrations at *D6S311*. Markers *D6S978* and *D6S1564*, which are located close to *D6S311*, showed allelic losses and imbalances in 57% and 50%, respectively. Allelic losses of *D6S441* on 6q26 (60%) were confirmed by detection of monoallelic complete deletions of *D6S1599* in 58% (7 of 12 informative cases). Results of deletion mapping are given in Fig. 3. As observed before, complete allelic losses (41%) dominate over imbalances (16%).

DISCUSSION

In the cases analyzed in the presented study, H-RS cells lost an average of ~46% of their alleles, which is comparable with several solid tumors that have lost 25% to 50% of their alleles as they progressed (32). The loci most frequently altered in our study were *D6S311* and *D6S441* on chromosome 6q followed by losses at *D3S1580*, *D12S98*, and *D2S391* on chromosomes 3q, 12p, and 2p, respectively. Genetic aberrations were found to be highest at *D6S311* on 6q25 in H-RS cells in 11 of 14 cHL cases. Although 6q aberrations are recurrent in 10% to 20% of cHL (19), allelic losses and imbalances of a defined region on chromosome 6q in ~80% of cases have not been described. To our knowledge, no other genetic marker has been described to be altered in H-RS cells with a similar consistency.

In lymphomas, deletions of 6q were detected at a frequency of 7% to 42%, depending on the subtypes and the method applied (31, 33–36). One report points toward a correlation between distinct lymphoma subtypes and specific deletions on 6q21, 6q23, and 6q25–27 (34), but the specificity of these findings could not be confirmed in an independent FISH analysis (37). Nevertheless, a tumor-suppressive potential of chromosomal regions on 6q, particularly on 6q25 has been shown *in vitro* and *in vivo* (38, 39). Tumor-suppressor genes like *LATS-1* (40) or *hZAC* (41) located within this chromosomal region thus might play a role in the pathogenesis of several tumors and possibly also in cHL.

In H-RS cells, other microsatellite markers like *D3S1580* and *D2S391* are deleted recurrently as well. *D2S391* displays LOH in 57% of informative cases and is located at 2p23.1. Several candidate tumor suppressor genes are located in the vicinity of that chromosomal region, including the mismatch repair gene *hMSH2*. This gene is of crucial importance for the correct functioning of the mismatch repair machinery. A defect of *hMSH2* or other mismatch repair genes causes microsatellite instability and might contribute to malignant transfor-



Cases / Marker	1739	1584	1507	L1236	1573	1858	1476	1566	1409	1494	1361	1414	1404	1622	1412	1454	LOH (%)
D6S1709		ni			ni	●			ni		●				ni		44
D6S1592				ni					●			ni		ni	ni	ni	29
D6s977	●		ni	ni		ni		●	●	ni	ni					ni	44
D6S308		ni	ni					ni				ni				ni	60
D6S310		ni				●	ni										33
D6s978	ni				ni		ni	●		ni	ni	ni	●	ni	ni		29
D6S311					●	●					●		ni				57
D6s1564					●	●							ni	ni			33
D6s440				ni	ni			ni	ni			ni	ni	ni			44
D6s1599	●	ni				ni							ni				58
D6S441				ni	ni		ni				ni		●		ni		60
D6s1590		ni		ni					ni		ni		●	●	ni		25
D6s281		●		ni			ni	ni						ni	ni		9
LOH (%)	64	71	64	100	17	13	71	56	0	38	14	40	0	75	29	20	

Fig. 3. 6q ideogram and results of allelotyping for detection of LOH in single-pooled H-RS cells. Cytogenetic position of microsatellite markers is indicated to scale according to GeneMap '99 (www.ncbi.nlm.nih.gov/genemap99/) and the ensembl database (www.ensembl.org). *Filled boxes*, complete LOH; *filled boxes with open circles*, imbalances; *hatched boxes*, no LOH; open boxes, no PCR product. *ni*, not informative.

mation of H-RS cells. In recent studies, however, microsatellite instability was not detected in pooled H-RS cells, and hMSH2 has been demonstrated to be expressed in cases of cHL (15, 16).

In this study, whole cells were isolated from cytospins (instead of frozen sections) and subsequently pooled to exclude loss of chromosomal material. Because most allelic losses were complete, genomic imbalance with amplification of the second allele could explain the microsatellite pattern detected in these cases. However, there are cases showing a pattern indicative of genomic imbalance. This imbalance might represent an incomplete allelic loss of one of the alleles or an amplification of the other allele. In these cases, incomplete losses may be explained by the presence of H-RS subclones lacking this particular allelic loss.

In summary, here we describe for the first time a recurrent aberration of a genetic marker in cHL, *i.e.*, the microsatellite marker *D6S311* located on chromosome 6q25, which is altered in ~80% of the cases analyzed. The indicated region on 6q harbors several tumor-suppressor genes that might be involved in the malignant transformation of H-RS cells.

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