BRIEF COMMUNICATION

Clonal Loss of Heterozygosity in Microdissected Hodgkin and Reed-Sternberg Cells

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The typical Hodgkin and Reed-Sternberg (HRS) cells are thought to represent the malignant cellular elements of Hodgkin's disease (HD). The detection of immunoglobulin gene rearrangements in HRS cells indicates that they are clonally derived from B cells *(1–8)*, but immunogenotyping, as such, does not provide any information on specific gene alterations possibly involved in the molecular pathology of HD. In many tumors, highly informative polymorphic DNA markers may identify loci harboring clonal loss of heterozygosity (LOH) and thus help to trace tumor suppressor genes *(9,10)*. Although in HD, cytogenetic data suggest that nonrandom chromosomal deletions may occur at several loci, including 1q42, 4q26, and others, very little (if anything) is known about clonal LOH in HRS cells at the molecular level *(11–15)*. We, therefore, set out to study microdissected HRS cells from classical types of HD at candidate loci for LOH with a highly sensitive microsatellite polymerase chain reaction (PCR) assay.

In seven patients with classical HD, HRS cells and surrounding cells including bystander lymphocytes were laser microdissected from formalin-fixed, paraffin-embedded tissue sections (Table 1). From a patient with nodular-sclerosing HD (patient 6), samples were taken at presentation (6a) and at relapse (6b). In two patients (patient 6 and patient 7, a patient with mixed cellularity-type HD), frozen sections of lymphoma tissue yielded high-molecular-weight control DNA from microdissected cell populations. Buccal smears provided constitutional high-molecular-weight DNA in patients 5 and 6 *(16,17)*. Patients gave their informed consent to include their material in this study. For molecular analyses, tetranucleotide repeat microsatellites were selected through the Genome Data Base at loci with a high frequency of chromosomal deletions in HD, which were predicted by the cytogenetic literature *(11–15)*, and located at 1q42 (D1S517), 4q26 (D4S2301), 9p23 (D9S254), and 11q23 (D11S1294). Their PCR primer sequences are available in the Genome Data Base (http://www.gdb.org). A highly sensitive seminested PCR assay included a first round that used the primers indicated above. In a second PCR, one of the primers in each pair was replaced by an internal forward primer (Fint) or an internal reverse primer (Rint): 1q42 (D1S517Rint) 5′-CATGTGTCCATCAATGGTAG-3′; 4q26 (D4S2301Fint) 5′-GATGAGTGCTTAGACCATAGTA-3′; 9p23 (D9S254Rint) 5′-GTCTCCAATGCATGANCTT-3′; and 11q23 (D11S1294Rint) 5′-CTGGTTTGCTTTCCCTTTCTT-3′ (software = "Primers! For the WWW"; http://www.williamstone.com) (Fig. 1, A). Allelic dropout during amplification of polymorphic microsatellite fragments may randomly affect either allele mimicking LOH. To exclude this pitfall, pooled samples of 10 purified HRS cells from a given patient were amplified *(18,19)*. Ten picograms of DNA (corresponding to the DNA content of a single diploid cell) was consistently detected by our PCR assay. We also used a seminested PCR *(1,3,20–22)* to examine HRS cells from patients 3, 4, 6, and 7 for clonally rearranged immunoglobulin heavy-chain genes created through joining of variable, diversity, and joining immunoglobulin gene regions (V-D-J joining). The primers were a framework III immunoglobulin gene VH primer 5′-ACACGGC(C/T)(G/C)T(G/A)TATTACTGT-3′ and a consensus JH primer *(21)* 5′-ACCTGAGGAGACGGTGACC-3′. The seminested primer was from VLJH sequences (5′-GTGACCAGGT(N)CCTTGGCCCCA-3′) *(22)* (Fig. 1, B).

DNA extracted from buccal smears and from bystander lymphocytes showed constitutional individual-specific microsatellite patterns in all patients (Fig. 1, A). Of 259 samples of HRS cells microdissected from formalin-fixed tissue, 59 (23%) yielded a detectable microsatellite PCR product. Mock picks from tissue sections cleared of cellular material were consistently negative by PCR analysis. All patients except patient 5 reproducibly showed clonal LOH in HRS cells at one to three different microsatellite loci (Table 1). LOH was seen at all loci, but the locus most frequently altered was D4S2301 (4q26; four of five informative patients). High-molecular-weight DNA from fresh HRS cells and corresponding degraded DNA from formalin-fixed tissue yielded identical individual-specific microsatellite band patterns (Fig. 1, A). None of the HRS cell samples showed extra microsatellite bands *(23)*. Patients 3, 4, 6, and 7 showed clonal immunoglobulin heavy-chain gene configurations in HRS cells and polyclonal patterns in bystander lymphoid cells (Fig. 1, B). In patient 6, a common rearranged immunoglobulin heavy-chain gene band in HRS cells, observed at presentation (6a) and at relapse (6b), indicated that the initial monoclonal population of HRS cells had relapsed. Our immunogenotype data from formalin-fixed HRS cells are in keeping with published data that HRS cells are mostly monoclonal B-cell populations *(1–8,20,24)*.

The detection of LOH has several implications in HD. The patients' buccal mucosa and bystander lymphocytes showed constitutional microsatellite patterns indicating that LOH in HRS cells was an acquired specific genetic feature of HD. To the best of our knowledge, this is the first report on microsatellite PCR detection of clonal LOH in microdissected HRS cells. In contrast to our approach, classical cytogenetics permit the study of mitotic cells only and usually do not permit the identifi-

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Table 1. Pathology of Hodgkin's disease (HD) samples and results of microsatellite polymerase chain reaction (PCR) analysis in microdissected Hodgkin and Reed-Sternberg (HRS) cells*

Samples							Microsatellite typing			
Histologic diagnosis (sample No.)	Sex/age, y	Stage†	CD15/30	EBER	LMP	β/gp220	D1S517 1q42	D4S2301 4q26	D9S254 9p23	D11S1294 11q22–23
Nodular sclerosing HD (1)	M/43	III	+/+	+	+	+	ni	1	nd	ni
Mixed cellularity HD (2)	F/43	III	+/+	+	+	+	2	1	ni	1
Nodular sclerosing HD (3)	F/16	III	+/+	−	nd	+	2	1	ni	1
Mixed cellularity HD (4)	F/74	III	+/+	+	+	+	1	1	1	2
Nodular sclerosing HD (5)	F/38	III	−/+	nd	nd	nd	ni	nd	ni	nd
Nodular sclerosing HD (6a)	F/69	III	−/+	nd	nd	+	2	nd	nd	nd
Nodular sclerosing HD in relapse (6b)		—	−/+	nd	−	nd	2	2	1	2
Mixed cellularity HD (7)	F/71	II	+/+	nd	−	nd	1	ni	ni	1

*1 = patient constitutionally informative and loss of one microsatellite allele (loss of heterozygosity) in HRS cells (data verified twice); 2 = patient constitutionally informative, two alleles retained in HRS cells; + = immunostaining done and positive; − = immunostaining done and negative; EBER = Epstein-Barr virus-encoded RNA; LMP = Epstein-Barr virus latent membrane protein; M = male; F = female; nd = molecular analysis not done due to lack of suitable material or failure to obtain PCR amplicons; and ni = patient not informative.

†Cotswold staging classification.

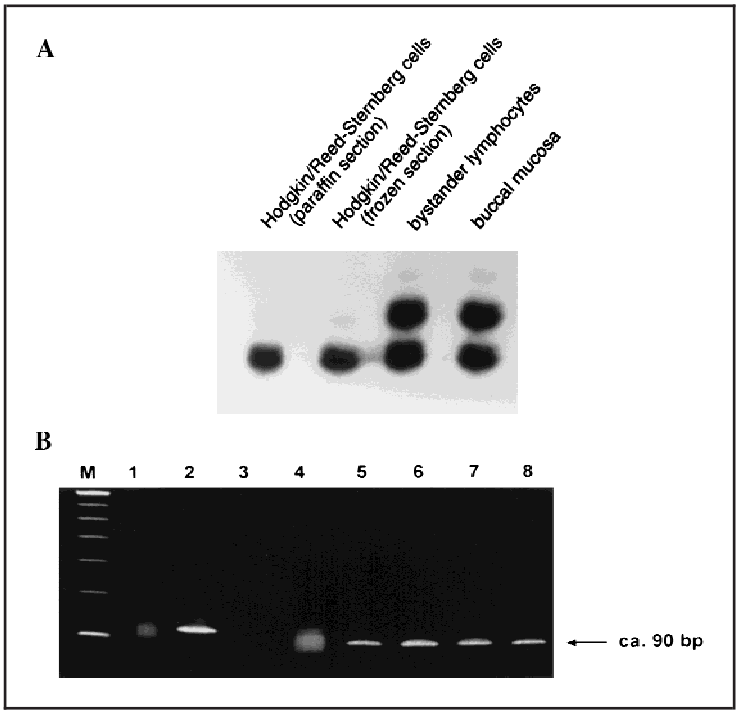

Fig. 1. A) Microsatellite detection of loss of heterozygosity (LOH) at D1S517 (1q42) in Hodgkin and Reed-Sternberg (HRS) cells from patient 7, a patient with a mixed cellularity-type Hodgkin's disease (HD). DNA from bystander lymphoid cells and from buccal mucosa shows a constitutionally heterozygous microsatellite pattern (two allelic bands). HRS cells isolated from formalin-fixed, paraffin-embedded sections and fresh-frozen sections show a single lower allele, indicating LOH at 1q42. $CD30^+$ HRS cells as well as bystander lymphocytes and other infiltrating cells in HD lymphoma sections were microdissected under an inverted laser microscope (P : A : L : M; GmbH, Wolfratshausen, Germany) *(17)* and were subjected to a sensitive seminested microsatellite polymerase chain reaction (PCR). The first PCR round was run on a Thermal Cycler 480 (Perkin-Elmer, Rotkreuz, Switzerland) with 2 m*M* $MgCl_2$, all four deoxynucleoside triphosphates (dNTPs, each at 0.1 m*M*), 1 U of hot-start *Taq* polymerase (AmpliTaq Gold; Perkin-Elmer, Rotkreuz, Switzerland), and 1 μ*M* of each primer under the following conditions: Initial denaturation was at 95 °C for 15 minutes, 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds**,** and the final elongation step was at 72 °C for 7 minutes. One microliter of a 1 : 20 dilution of first-round PCR amplicons was subjected to a second round of PCR on a Perkin-Elmer 9600 Thermal Cycler in 1.5 m*M* $MgCl_2$, all four dNTPs (each at 0.1 m*M*), 0.5 U of Boehringer® *Taq* DNA polymerase, and 1 μ*M* of each primer under the following conditions: Initial denaturation was at 94 °C for 5 minutes, 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds, and the final elongation step was at 72 °C for 7 minutes. If a PCR band was identified on agarose gel electrophoresis, 1 μL of a 1 : 20 dilution of the respective first-round PCR sample was subjected to a second round of PCR by use of the appropriate internal ^{32}P-labeled primer, as described above. Labeled PCR fragments were subjected to electrophoresis on denaturing 6% polyacrylamide-sequencing gels and subjected to autoradiography at −70 °C for 2–12 hours as appropriate *(19)*. LOH seen in a given patient at a particular locus was confirmed by at least one or several repeat analyses of cells separately harvested from adjacent tissue sections. **B**) Immunoglobulin heavy-chain gene PCR analysis from patient 6, a patient with nodular sclerosing HD. **Lane 1**—peripheral blood lymphocytes from a healthy donor; **lane 2**—Nalm6 (monoclonal B-cell leukemia cell line with rearranged immunoglobulin heavy-chain genes); **lane 3**—Calu1 (lung cancer cell line with germline immunoglobulin heavy-chain genes); **lane 4**—bystander lymphoid cells microdissected from paraffin-embedded HD sections; **lanes 5** and **6**—pooled microdissected HRS cells obtained at presentation; and **lanes 7** and **8**—single HRS cells obtained at relapse. M = molecular weight marker (100-base-pair [bp] DNA ladder). HRS cells at presentation and at relapse show a common rearranged immunoglobulin heavy-chain gene band of about 90 bp. Lymphoid cells in lanes 1 and 4 yield multiple rearranged immunoglobulin heavy-chain gene bands indicating polyclonal B-cell populations. The first PCR round was run in 2.5 m*M* $MgCl_2$, all four dNTPs (each at 0.2 m*M*), 1 U of hot-start *Taq* polymerase (AmpliTaq Gold; Perkin-Elmer), and 1.3 μ*M* of each immunoglobulin gene primer under the following conditions: Initial denaturation was at 95 °C for 15 minutes; 10 cycles with a stepwise decrease of annealing temperatures from 65 °C to 56 °C were followed by 30 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 45 seconds and a final elongation step at 72 °C for 7 minutes. The second-round PCR used 1 μL of a 1 : 200 dilution of the first-round PCR product as template and was run under the conditions described above, except for using 2 m*M* $MgCl_2$ and running 40 cycles with an annealing temperature of 55 °C. The final PCR products were separated on 4%–20% polyacrylamide gradient gels and visualized by ethidium bromide staining.

cation of the lineage of cells being karyotyped. The detection of LOH restricted to HRS cells is consistent with the view that they are clonally derived from a common single cell of origin *(16,25–27)*. The probability in a given patient that all HRS cells tested would have lost the same allelic microsatellite band independently or by chance is .002 (i.e., 0.5^{n-1}, where n is the number of HRS cells examined) and, therefore, is low *(28)*. Our findings thus confirm and extend molecular immunogenotype data on the clonality of HRS cells in classical HD with a molecular marker system unrelated to the configuration of immunoglobulin genes.

The detection of LOH in tumor cells at a particular locus indicates clonality and by the same token points to a site in the genome possibly harboring inactivated tumor suppressor genes. In non-Hodgkin's lymphomas, inactivation of tumor suppressor genes has been described at the p53 gene or the $p16^{INK4a}$ locus. Our work now shows that in classical HD, clonal LOH may be present at loci, such as 1q42, 4q26, 9p23, 11q22–23, and possibly others. The dense genomic map of microsatellite markers now available will help to narrow down such loci as a next important step for the eventual cloning of tumor suppressor genes operative in the molecular pathology of HD.

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