

Clonal Relationship of Classical Hodgkin Lymphoma and Its Recurrences

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Abstract

Purpose: We investigated whether the first and all subsequent manifestations of Hodgkin lymphoma (HL) in a patient are clonally related.

Experimental Design: We identified a collective of 20 patients with sometimes multiple HL recurrences. Relapses were classified as early, that is, within twelve months (eight events in seven patients) or as late, that is, later than one year after the previous neoplasm (24 events in 17 patients). Hodgkin and Reed–Sternberg cells were microdissected after CD30 staining using laser capture technique. Immunoglobulin heavy chain (*IgH*) gene fragment lengths were analyzed after DNA preamplification, applying consensus FR3 and J primers by ABI 310 Genetic Analyzer. Sequencing of the amplified *IgH* products was carried out by ABI 3130 and 3730XL Genetic Analyzer. Epstein-Barr virus (EBV) association was assessed by EBV early RNA and LMP1.

Results: Three cases with early relapses after a first HL diagnosis were clonally related to the initial tumor, whereas three of four patients with early relapses after a first or second relapse were not, which was accompanied by change of EBV association in one case. Six patients presenting with late relapses were clonally unrelated, which was accompanied by change of phenotype in two cases and change of EBV association in one case. Two samples from recurrent tumors of the same patient could be successfully sequenced. These two late relapses were clonally unrelated by *IgH* fragment length and sequencing analysis.

Conclusions: Recurrent HL, especially those accompanied by an EBV-association switch or after a relapse, can represent an unrelated novel neoplasm. Our finding might play a role in clinical decision making. *Clin Cancer Res*; 17(16); 5268–74. ©2011 AACR.

Introduction

Classical Hodgkin lymphoma (HL) is a highly curable malignancy when treated with multimodal therapy (1). However, up to 30% of patients fail to respond or suffer a relapse after initial therapy (2–4). Intensive chemotherapy with or without autologous stem cell support is now the treatment of choice for patients with relapsed chemosensitive HL (5, 6).

Hodgkin and Reed–Sternberg cells represent the tumor cell population of HL. Both B cell origin and monoclonality of Hodgkin and Reed–Sternberg cells have been

established (reviewed in ref. 7). It has also been shown that in composite lymphomas, consisting of an HL and a so-called "non-HL" (NHL), a clonal relationship between both components can exist (8–11), but studies on the clonal relationship of recurrent HL have so far been limited to case reports (12).

Our aim was to specifically analyze the clonal relationship of recurrent HL by several molecular techniques in addition to morphology, phenotype, and Epstein-Barr virus (EBV) association. The study reported herein assessed the clonal relationships of the largest number of recurrent HL analyzed to date.

Materials and Methods

Patients

Patients with recurrent HL were identified from the archives of the Institutes of Pathology in Basel, Bern, and Liestal (all in Switzerland). Tissue samples of the identified patients were included in our analysis if adequately preserved for immunohistochemical and molecular analyses. Clinical and follow-up data were obtained by reviewing the charts. Retrieval of tissue and clinical data were carried out according to data safety laws.

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Translational Relevance

Our finding that not all recurrent Hodgkin lymphomas (HL) are true "relapses" of the original malignant clone may be of clinical significance in that it raises questions about the current aggressive clinical therapies in such instances, suggesting that clonally unrelated relapsed HL might still qualify for less intensive therapeutic approaches. Moreover, our findings provide further evidence of the role of individual predisposition and of the possible presence of clonotypic lymphoma progenitor cells in the pathogenesis of HL.

Histopathology, immunohistochemistry, and *in situ* hybridization

All cases were reclassified according to the most recent WHO criteria (13). Four- μ m sections were cut from routinely processed, formalin-fixed, and paraffin-embedded specimens placed on adhesive-coated slides. The slides were immunohistochemically stained using an automated immunostainer (BenchMark XT). The following primary antibodies were utilized: CD30 (clone Ber-H2; catalogue no. 790-2926, Ventana, dilution as supplied), and LMP1 (clone CS1-4, catalogue no. CMC426, Cell Marque, dilution 1:25). No counterstain was used in case of the CD30 staining.

In situ hybridization for EBV early RNA (EBER) was carried out on deparaffinized and dehydrated sections. Chromogen-labeled oligonucleotides complementary to portions of the EBV-encoded early RNA transcripts (Inform probe, ref. 800-2842), were used according to the manufacturer's protocol. Positivity was defined as clear labeling of all Hodgkin and Reed-Sternberg cells per slide.

Microdissection and molecular analysis

Laser capture microdissection similar to that described previously was carried out (8). Briefly, uncovered and uncounterstained tissue slides stained for CD30 were utilized. CD30-positive cells were identified, separated by laser beam from the surrounding tissue, and catapulted into the lid of an Eppendorf cup with aid of the PalmRoboSoftware 3.0 on a PALM MicroBeam-System (Zeiss).

DNA from the microdissected Hodgkin and Reed-Sternberg cells was extracted using a Genovison extraction kit (Qiagen), as described (8). Pre-amplification was carried out using the REPLI-g FFPE Kit (Qiagen), according to the manufacturer's instructions.

PCR analysis of immunoglobulin heavy chain (*IgH*) gene rearrangements was carried out utilizing consensus FR3 and J primers, as published previously (14). To verify results, a second PCR with a different set of primers was carried out. This second experiment utilized primers designed to give 4 bp shorter PCR products than the products from the first PCR (primer sequence: for *IgH* FR3: FR3C ACACGGCYGTGTATTACTGT labeled with

6-FAM, and for *IgH* J: JOIN A - CCTGAGGAGACGGT-GACC, JOIN B - CCTGAAGAGACGGTGACCA, JOIN C - CCTGAGGAGACAGTGACCAG). Examination of PCR products was carried out with the high-resolution fragment length analysis (ABI 310 Genetic Analyzer, Applied Biosystems). Clonality was assumed by the demonstration of a peak in the initial PCR run and a peak belonging to a PCR product, which was 4 bp shorter in the verification PCR than in the initial PCR. A shift of the PCR products of more than 1 bp between the cases was considered to indicate a clonally unrelated event.

Sequencing of the amplified *IgH* gene FR3 region products was carried out, both in our institution and by a commercial company (Microsynth; www.microsynth.ch) in 45 samples of all patients, where DNA was available after *IgH* fragment length analysis. Sanger sequencing was carried out with the laser-induced fluorescence detection system ABI 3130 and 3730XL Genetic Analyzer and the corresponding software (Applied Biosystems).

Results

Patients

A total number of 20 patients with recurrent HL were collected from the Institutes of Pathology in Basel ($n = 12$), Bern ($n = 3$), and Liestal ($n = 5$). In 5 patients it was not possible to evaluate the initial HL, but because it was possible to assess the following recurrences, these cases were not excluded (no. 11, 15–17, 20). The patients with evaluable tumors were comprised of 12 males (average age at diagnosis 32; range: 5–67) and 8 females (average age at diagnosis: 44; range, 20–86).

An overview of all cases with the number and chronology of recurrences is presented in Figure 1 and Table 1. Twelve patients had 1 recurrence (no. 1, 2, 4–10, 12, 13, and 18), 5 patients experienced 2 recurrences (no. 3, 11, 16, 19, and 20), 2 patients had 3 recurrences (no. 14 and 17), and 1 patient had 4 recurrences (no. 15). There were early relapses in 7 patients [no. 1–3, 11, 14, 15, and 20; including 2 subsequent early relapses (no. 3)], that is, 3 early relapses after first HL diagnosis (no. 1–3), 5 early relapses after a relapse (no. 3, 11, 14, 15, and 20), and late recurrences in 17 patients (no. 4–20). In 1 case (no. 5), the recurrent HL was preceded by a follicular lymphoma, grade 2, which had occurred 8 years before the first diagnosis of HL.

Two further cases were also analyzed: 1 patient (no. 21; male; age at diagnosis: 45) had originally presented with an HL (mixed cellularity subtype), followed by a diffuse large B-cell lymphoma (DLBCL) 2 years later. The second patient (no. 22; female, age at diagnosis: 69) had a DLBCL initially that recurred as an HL (mixed cellularity subtype) 6 years later.

All patients were treated with intention to cure. At the point of last clinical contact, out of all 22 patients, 17 are still alive, whereas 5 have died of their disease. Clinical details, including the type of therapy administered, are presented in Table 1.

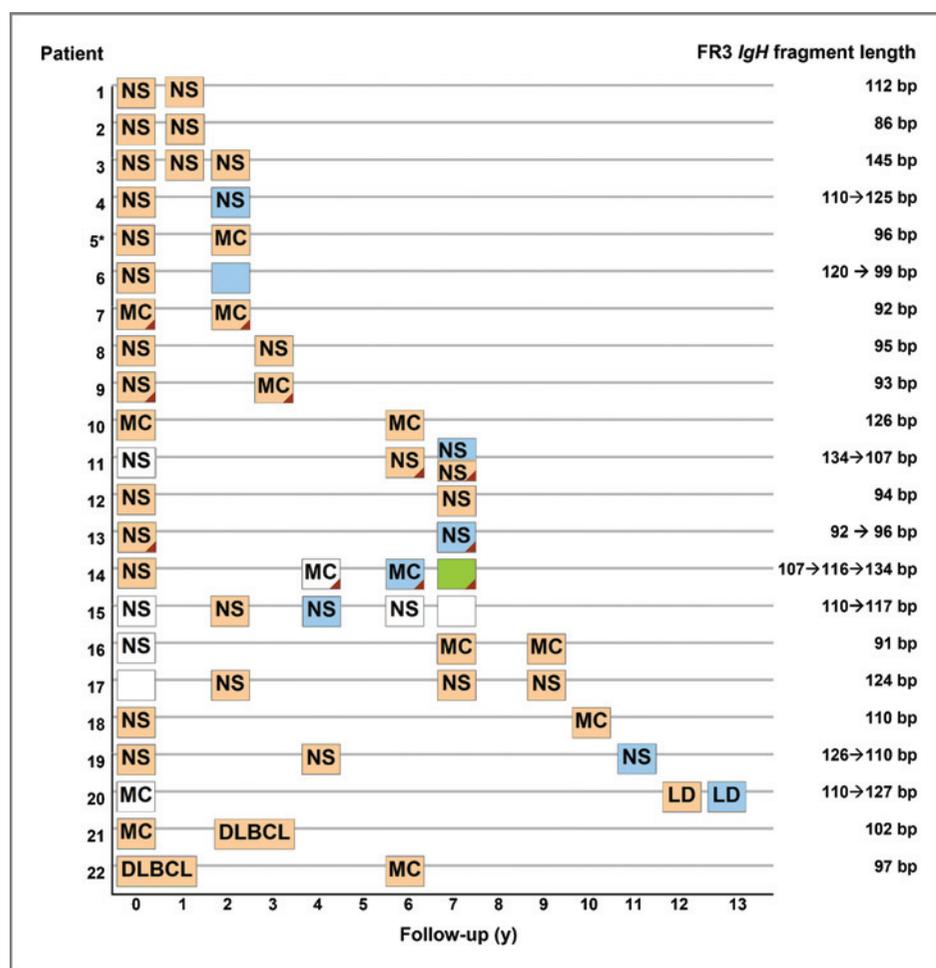


Figure 1. Chronological overview of HL relapses. Change of clonal relationship is highlighted by different colors. Brown triangles indicate positivity for LMP1/EBER. Unlabeled rectangles indicate morphologically unclassifiable HL cases. Uncolored rectangles indicate PCR analysis failures. Abbreviations: NS, nodular sclerosing subtype of HL, MC, mixed cellularity subtype of HL; LD, lymphocyte depleted subtype of HL. *, indicates the patient with preceding colonally unrelated follicular lymphoma.

Histopathology, immunohistochemistry, and *in situ* hybridization

Sixteen HL presented initially as nodular sclerosing (no. 1–6, 8, 9, 11–16, 18, and 19), and 4 cases as mixed cellularity subtype (no. 7, 10, 20, and 21). It was not possible to get information on the initial subtype in 1 case (no. 17). A change of subtype was observed in 6 cases (no. 5, 9, 14, 16, 18, and 20).

EBV association was evaluable in all cases: in 15 patients, both initial disease and recurrence(s) were EBER and LMP1 negative (no. 1–6, 8, 10, 12, and 15–20). A switch from EBER/LMP1 negativity to positivity was observed in 1 patient (no. 14). Interestingly, 1 patient (no. 11) who presented with simultaneous recurrences at 2 different anatomic regions had both an EBER/LMP1-positive and an EBER/LMP1-negative tumor at each site. The patient with recurrent HL preceded by follicular lymphoma (no. 5) and the 2 patients with composite DLBCL and HL (no. 21 and 22) were EBER/LMP1-negative in all tumors.

Microdissection and molecular analysis

Analysis of *IgH* fragment length was successful in all patients (Fig. 1). On average, it was possible to microdissect

350 Hodgkin and Reed–Sternberg cells per sample. In 2 cases (first relapse of no. 14 and third relapse of no. 15, both with irresolute PCR results), up to approximately 500 cells per case were microdissected.

In 3 samples (first and second relapse of no. 3 and fourth relapse of no. 15, the latter with irresolute PCR results), because of the small size of the diagnostic sample, the whole tissue section was used without microdissection.

By *IgH* fragment length analysis, all 3 early relapses after first HL diagnosis were clonally related to their initial tumor (no. 1–3), whereas 3 of 4 early relapses after a first or second relapse were not (no. 11, 14, and 20); 1 of the latter patients had 2 simultaneous clonally unrelated early second relapses (after first recurrence) at different anatomical sites and with divergent EBV association (no. 11). Six of 20 analyzable late relapses were clonally unrelated, which was accompanied by a switch of phenotype in 2 cases (no. 6 and 14) and a change of EBV association in 1 case (no. 14). All other late recurrences were clonally related to the initial HL (Fig. 2). In the case of recurrent HL preceded by a follicular lymphoma, both HL were clonally related to each other despite a switch of phenotype; however, the preceding follicular lymphoma was not

Table 1. Patient characteristics

Case no.	Sex/Age, y	Year of initial diagnosis	Stage	Therapy of initial disease	Therapy of first relapse	Follow-up (y)	Outcome
1	F/20	2006	II A	ABVD	DHAP, RT, BEAM + AUTO-SCT, ALLO-SCT	3	Alive
2	F/75	1992	II B	ABVD + RT	None	1	Dead
3	M/27	2001	II B	BEACOPP + RT	DHAP, RT, IMVP16 + AUTO - SCT	2	Dead
4	F/86	2006	III A	ABVD	Not available	2	Dead
5	M/67	2006	II A	ABVD	BEACOPPesc	3	Alive
6	M/18	2004	III AS	BEACOPP	DHAP, BEAM + AUTO-SCT	4	Alive
7	M/5	2006	III A	OEPA + COPP + RT	Not available	3	Alive
8	M/21	2003	III B	BEACOPPesc	Not available	4	Alive
9	M/53	1997	IV B	COPP-ABVD + RT	DHAP, EPOCH + AUTO-SCT	7	Alive
10	F/37	2002	II A	ABVD + RT	BEACOPPesc	8	Alive
11	M/20	1999	II A	ABVD + RT	DHAP, RT, BEAM + AUTO-SCT	10	Alive
12	F/48	2001	IV B	BEACOPPesc + RT	DHAP, BEAM + AUTO - SCT	7	Alive
13	F/39	1993	II A	ABVD	DHAP, EPOCH + AUTO-SCT	9	Alive
14	M/64	2002	III B	Not available	Not available	7	Alive
15	M/23	2001	III B	ABVD	BEACOPPesc	7	Dead
16	M/36	1999	II A	ABVD	BEACOPPesc	10	Alive
17	F/20	1997	IV B	MOPP-ABVD + BEAM + AUTO-SCT-RT	BEACOPPesc, DHAP + 2xALLO-SCT	13	Alive
18	M/20	1994	IV B	COPP-ABVD + RT	BEACOPPesc, DHAP + AUTO-SCT	15	Alive
19	M/33	1997	II A	BEACOPP + RT	DHAP + AUTO-SCT	12	Alive
20	F/22	1991	III B	BEACOPP + RT	BEACOPP, DHAP + AUTO-SCT	15	Clinical recurrence
21	M/45	2001	III B	ABVD	R-CEOP	8	Dead
22	F/69	2000	I A	CHOP + RT	ABVD	9	Alive

Abbreviations: ABVD, adriamycin, bleomycin, vinblastine, dacarbazine; ALLO-SCT, allogeneic stem cell transplantation; AUTO-SCT, autologous stem cell transplantation; BEACOPP, bleomycin, etoposide, adriamycin, cyclophosphamide, vincristine, procarbazine, prednisone; BEACOPPesc, BEACOPP escalated; BEAM, carmustine, etoposide, cytarabine, melphalan; CHOP, cyclophosphamide, adriamycin, vincristine, prednisone; COPP, cyclophosphamide, vincristine, procarbazine, prednisone; DHAP, cytarabine, cisplatin, dexmethasone; F, female; ICE, ifosfamide, carboplatin, etoposide; IMVP16, ifosfamide, methotrexate, etoposide; M, male; MOPP, mechlorethamine, vincristine, procarbazine, prednisone; OEPA, vincristine, etoposide, prednisone, adriamycin; R-CEOP, rituximab, cyclophosphamide, epirubicin, vincristine, prednisone; RT, radiotherapy.

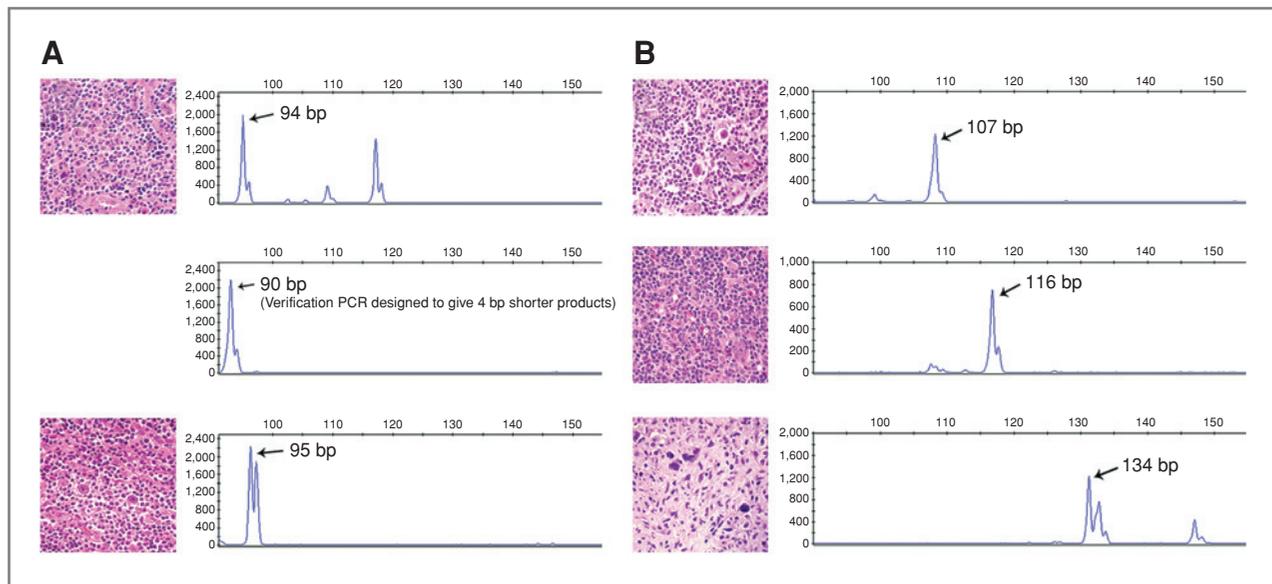


Figure 2. Clonal peaks in (A) clonally related (patient no. 12) and (B) clonally unrelated (patient no. 14) relapsing HLs. Inserts illustrate the morphology of the corresponding samples. A, clonal peaks at 94 bp in the first PCR run and at 90 bp in the verification PCR run, designed to give shorter products, are seen in the first 2 graphs from the initial HL sample of patient no. 12. A clonal peak at 95 bp corresponding to a clonally related neoplasm is seen in the third graph from an HL relapse, which occurred 7 years later. B, clonal peaks at 107, 116, and 134 bp in the corresponding PCR runs are seen in the 3 graphs from the initial diagnosis, and second and third relapses of patient no. 14, which occurred 6 and 7 years later.

related to the HL. The 2 composite cases, initially presenting as HL and relapsing as DLBCL or vice versa, showed clonal relationships between both entities.

Sequencing of the amplified *IgH* gene FR3 region products was carried out in 45 samples of patients no. 1 to 20, but yielded interpretable results confirming *IgH*-derived gene sequences in 10 samples from 9 patients (no. 1, 4, 5, 8, 11, 14, 15, 18, and 19) because of too low DNA amounts (picogram range instead of required nanogram range). However, 2 samples from recurrent tumors of a same patient (no. 15) could be successfully sequenced. These 2 late relapses were clonally unrelated, both by *IgH* fragment length and sequencing analysis (Fig. 3).

Summary and Conclusions

To our knowledge, this is the largest study to date assessing the clonal relationship of recurrent HL. We provide strong evidence—supported in 1 case also by gene sequencing—that not all cases of recurrent HL are true clonal "relapses" of the original malignant clone. There are 3 findings that support the existence of a second nonrelated neoplasia: (i) change of histologic subtype, (ii) altered EBV association, and, most important, (iii) clonal diversity as shown by different *IgH* fragment lengths in 8 cases as well as different *IgH* gene sequences in 1 case.

Change of morphologic subtype has been previously noted in recurrent HL, but has not been taken as a sign of lacking relationship because treatment might change the appearance of the lymphoma (15). This hypothesis is supported by our finding of molecular kinship in

morphologically different tumor recurrences (e.g. no. 5, 9, and 18).

Assessment of EBV association has been used to study clonal relationship of recurrent HL, and these authors found a high correlation between expression of LMP1 in primary and recurrent HL (15). We observed a change of EBV association (EBER and LMP1 expression) in 2 patients (no. 11 and 14), which is in accordance to the literature (16). Although, at least in our series, both patients with an EBV association switch had clonally unrelated HL relapses,

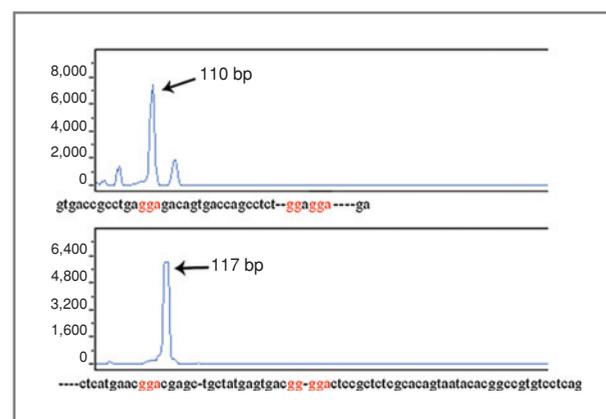


Figure 3. Different immunoglobulin heavy chain fragment lengths and different *IgH* gene sequences in a first (top) and second relapse (bottom) of patient no. 15. A peak at 110 bp is seen in the first relapse, whereas a peak at 117 bp appears in the second relapse, which occurred 2 years later. Sequencing of the amplified *IgH* gene FR3 region products of the samples is shown below the graphs. Identical base sequences are highlighted in red.

a positive or negative correlation of EBV association will not prove beyond doubt the kinship of the tumors, particularly considering the possibility of EBV "hit and run" mechanisms in HL (11, 17).

Analyzing *IgH* rearrangements seems to be the most accurate way to determine the clonal relationship of a recurrent lymphoma. In HL, however, the scarcity of Hodgkin and Reed–Sternberg cells is a major drawback. A previous study focusing on HL with numerous Hodgkin and Reed–Sternberg cells showed that 6 of 7 cases contained clonally related rearranged *IgH* or light-chain genes (16). However, the authors analyzed whole samples in all cases rather than, as we did with the exception of 3 samples, laser-microdissected Hodgkin and Reed–Sternberg cells. In addition to these 3 samples, of which one was not analyzable and 2 showed clonal relationship to the microdissected initial diagnosis, and thus did not blur our results, 1 potential limitation of our study should be addressed; namely, the low amount and poor quality of the DNA (because of scarcity of Hodgkin and Reed–Sternberg cells, small core needle biopsies obtained in relapsing HL, application of laser beam for microdissection, and the archival nature of the samples), which though permitting result interpretation with the sensitive methodology of *IgH* fragment length analysis, allowed successful sequencing (more specific methodology) only in 10 samples of 9 patients. Importantly and supporting the validity of our results, in all these 10 instances sequencing confirmed *IgH*-derived genetic material. Yet, in 1 matching patient with 2 recurrences (no. 15), whose probes were analyzable by both methodologies, both results were concordant, that is, clonally unrelated HL relapses (Fig. 3), suggesting reliability of the platforms used. Furthermore, although somatic mutations are almost always detectable in the tumor cells of nodular lymphocyte predominant HL, this seems not to be a genuine feature of Hodgkin and Reed–Sternberg cells of classic HL (18). Thus, ongoing mutations can probably not explain the observed *IgH* length changes in our bona fide clonally unrelated cases, supporting our assumption of existing cases of clonally unrelated relapsing HL.

Our findings of clonally unrelated relapses raise 2 important issues. First, this might be of clinical importance because relapsing HL are treated aggressively without regard to the possibility of dealing with a second, newly developed, tumor. Thus, our findings may merit consideration in future clinical studies to determine if a less intensive treatment might be justifiable in "recurrent" clonally unrelated HL.

Second, the underlying mechanism of HL relapses needs to be defined. It is possible to hypothesize the existence of Hodgkin and Reed–Sternberg cells, which

are not altogether eliminated by therapy. Hodgkin and Reed–Sternberg cells are known for their defective cell cycle and apoptosis regulation, which enables them to escape therapy (reviewed in ref. 7), a theory which is supported by our finding of clonally related recurrences. Another hypothesis focuses on the existence of tumor stem cells in HL. Such cells represent a small, but significant, population in tumors that possess both tumor-initiation and self-renewal capacity; tumor stem cells have been identified in several human malignancies (19) and are suspected in HL (11). Recently, the existence of a clonotypic lymphoid cell population that morphologically does not resemble Hodgkin and Reed–Sternberg cells has been proven (20); these cells may give rise to a clonally related recurrence of HL as well. In support of the possibility of the existence of tumor stem or precursor cells in HL, we and others have shown that a clonal relationship can exist even between an HL and a metachronous NHL in the same individual (reviewed in ref. 21). Both genetic and environmental factors may predispose an individual to develop an HL; the relative risk of HL being highest (approximately 100-fold) in monozygous twins (22–25). Such predisposing factors might also increase the risk for a second clonally unrelated HL in patients with "recurrent" disease, as observed also in our cohort, especially with second and third recurrences after a relapse. It is tempting to hypothesize that such individuals may be prone to develop an HL, and that environmental factors, including the adverse effects of previous intensive treatment, might enhance this predisposition.

In summary, this is the largest study we are aware of to date, evaluating at the genetical level the clonal relationship of recurrent HL. By analyzing *IgH* fragment lengths, we provide strong evidence in 8 patients that not all "relapses" are clonally related to the primary neoplasm. In one patient, we further support this theory by sequencing of the *IgH* gene. These findings are interesting both in regard to different theories on relapsing HL and might have a practical implication on treatment strategies needing further evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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