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Hodgkin's disease variant of Richter's syndrome in chronic lymphocytic leukaemia patients previously treated with fludarabine

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Summary

The transformation of chronic lymphocytic leukaemia (CLL) into large-cell lymphoma (Richter's syndrome, RS) is a well-documented phenomenon. Only rarely does CLL transform into Hodgkin's lymphoma (HL). To further analyse the clinico-pathological and genetic findings in the HL variant of RS, we performed a single-institution study in four patients, who developed HL within a mean of 107 months after diagnosis of CLL. All were treated with fludarabine. Three cases were Epstein–Barr virus (EBV)-associated mixed cellularity (MC) HL, the fourth was nodular sclerosis (NS) HL without EBV association. The sites involved by HL included supra- and infradiaphragmal lymph nodes and the tonsils; stage IV disease was also documented. All patients presented with CLL treatment-resistant lymphadenopathies and B-symptoms. In two of the MC cases, molecular analysis performed on CLL samples and microdissected Hodgkin and Reed–Sternberg cells (HRSC) suggested a clonal relationship, while in NS no indication of a clonal relationship was detected. In summary, HL can occur in CLL patients at any site, up to 17 years after initial diagnosis, especially after treatment with fludarabine. The majority present with B-symptoms and CLL treatment-resistant lymphadenopathy, are of the MC type, clonally related to CLL and might be triggered by an EBV infection.

Keywords: Hodgkin's disease, CLL, Richter's syndrome, fludarbine.

B-cell chronic lymphocytic leukaemia (CLL) is the most frequent form of non-Hodgkin's lymphoma (NHL) affecting adults in Tyrol (Fiegl *et al*, 2003). It arises from a mature lymphocytic clone and is characterized by progressive accumulation of long-lived, immune-incompetent neoplastic lymphocytes in the blood, bone marrow and lymphoid tissues with a distinct phenotype. The clinical course of CLL is extremely variable, with about 80–90% of patients being asymptomatic at initial diagnosis. One-third of patients progresses rapidly to an aggressive disease requiring immediate treatment; another third presents with an initially indolent disorder, but experiences disease progression within the next 5 years; and the remaining third suffers from long-standing disease that never requires therapy, eventually dying of causes unrelated to CLL (Dighiero & Binet, 2000). Transformation to a high-grade lymphoma, called Richter's syndrome (RS), occurs in approximately 3% to 10% of CLL patients, with most cases being classified as secondary diffuse large B-cell lymphoma (DLBCL) (Giles *et al*, 1998). This form of RS in CLL patients appears to be independent of disease stage, type, or response to therapy and is often associated with a deterioration in patient's clinical symptoms: lymph node enlargement, B-symptoms and sometimes hepatosplenomegaly (Cohen *et al*, 1987). The prognosis of this variant of RS is quite poor and patients usually die within months of transformation, despite treatment.

Only a handful of reports on Hodgkin's lymphoma (HL) manifesting as a variant of RS, originally described by Richter (1928) and called 'reticular cell sarcoma', have been published, particularly in patients treated with purine analogues (Choi & Keller, 1981; Brecher & Banks, 1990; Shin *et al*, 1993; Rubin *et al*, 1994; Butts *et al*, 1995; Weisenberg *et al*, 1995; Fayad *et al*, 1996; Juneja *et al*, 1999; Kanzler *et al*, 2000; Adiga *et al*, 2003; Nemets *et al*, 2003). The frequency of CLL transformation into HL varies between 0.5% and 2.3% (Fayad *et al*, 1996; Keating *et al*, 1998). Epstein–Barr virus (EBV) is frequently

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detected in Hodgkin and Reed–Sternberg cells (HRSC) of the HL variant of RS, suggesting that EBV infection may play an important pathogenetic role (Momose *et al*, 1992; Tsang *et al*, 1993). In RS with a DLBCL component, the large-cell lymphoma and the CLL component have been found to share a common clonal origin in roughly half of the cases (Giles *et al*, 1998). However, only a limited number of cases of the HL variant of RS have been investigated for a clonal relationship between the small lymphocytic component and HRSC. These were found to be clonally related in some cases but not in others (Ohno *et al*, 1998; Kanzler *et al*, 2000; van den Berg *et al*, 2002; de Leval *et al*, 2004).

To further analyse the medical history, pathological, virological and molecular findings of patients with the HL variant of RS, we performed a single-institution study in four cases. The clonal relationship between CLL and HRSC was investigated in micromanipulated single cells.

Materials and methods

Patients and histology

All four cases of HL variant of RS diagnosed between 2000 and 2004 at the Department of Pathology of Innsbruck Medical University were included. The cases were classified according to the World Health Organization criteria (Stein *et al*, 2001). All patients were previously diagnosed, histologically (trephine bone marrow biopsy) and cytologically (smear and fluorescent-activated cell sorting), as suffering from B-CLL. The clinical data were obtained by reviewing the charts.

Immunohistochemistry

The samples were immunohistochemically analysed using an automated immunostainer (NexEs, Ventana, Tucson, AZ, USA). The streptavidin–biotin peroxidase technique with diaminobenzidine as chromogene was applied. The primary antibodies were diluted in a 1% solution of bovine serum albumin in phosphate-buffered saline (pH 7.4) and incubated for 30 min at room temperature. The primary antibodies, their dilutions and pretreatment conditions are listed in Table I.

EBV detection

To verify EBV infection of the HRSC in the samples, we performed *in situ* hybridization (ISH) for the EBV-encoded small RNAs (EBER) with the EBV probe *in situ* hybridization kit from Novocastra (Newcastle, UK) using the manufacturer's protocol.

Laser capture microdissection (LCM) of HRSC

Tissue sections 4-μm of each HL variant of RS, from patients 2, 3 and 4 (not from patient 1 because of lack of tissue), were mounted on slides. The slides were stained with an anti-CD30 antibody and left uncovered. High-sensitivity LCM CapSure caps (Arcturus, Hess. Oldendorf, Germany) were then mounted on the HRSC-rich areas of the prepared slides using the PixCell IIe system (Arcturus). Microdissection was performed by directing laser microbeats at the nuclei of the CD30-stained giant cells (90 mW; 1.5 ms; spot size 7.5 μm). Seventy-five to eighty HRSC per sample were collected.

Analysis of clonal relationship between HRSC and CLL

Formalin-fixed, paraffin-embedded trephine bone marrow biopsies obtained as early as possible before the appearance of clinical symptoms of HL transformation and containing CLL infiltrates without evidence of transformation were selected. The total DNA was extracted. This DNA, as well as the DNA from the microdissected HRSC of the HL variant of RS samples from the same patients, was extracted by incubating the probes with 95 μl digestion buffer G2 and 5 μl proteinase K (both from Qiagen, Hilden, Germany), in a thermomixer as suggested by the manufacturer (http://www1.qiagen.com/literature/protocols/pdf/MA11.pdf). For semi-nested polymerase chain reaction (PCR) analysis of B-cell clonality, two amplification runs were performed for 40 and 25 cycles, using 1% of the first-run product for the second run and applying primer sets (Sioutos *et al*, 1995) LJH 9 and FRIII A8 for the first run and VLJH 10 and FRIII A8 for the second run and the hot start *Taq* DNA polymerase (Qiagen) in a thermal cycler (Eppendorf, Hamburg, Germany). The primers

Table I. Antibodies and antigen retrieval techniques used.

Antibody	Dilution	Retrieval	Source
CD3	1:50	Citrate buffer pH 6, wet autoclave, 121°C, 5 min	Dako (Golstrup, Denmark)
CD5	1:150	Citrate buffer pH 6, wet autoclave, 121°C, 5 min	Novocastra
CD15	1:500	Citrate buffer pH 6, wet autoclave, 121°C, 5 min	Dako
CD20	1:700	Citrate buffer pH 6, microwave oven, 800 W, 10 min	Dako
CD23	1:40	Citrate buffer pH 6, wet autoclave, 121°C, 5 min	Novocastra
CD30	1:50	Citrate buffer pH 6, wet autoclave, 121°C, 5 min	Dako
CD45RA	1:500		Dako
CD79a	1:300	Citrate buffer pH 6, wet autoclave, 121°C, 5 min	NeoMarkers
LMP-1	1:1000	Pronase, 4 min	Dako
p53	1:50	Citrate buffer pH 6, wet autoclave, 121°C, 5 min	Dako

were synthesized with an automated DNA synthesizer (GenXpress, Maria Woerth, Austria). DNA integrity was assessed by amplifying the rhesus CE gene. The products were detected and compared using the restriction fragment length polymorphism in a polynat gel matrix (Elchrom Scientific, Cham, Switzerland).

Results

Clinical history, histology and immunohistochemical findings

The patients' clinical history is summarized in Table II. Importantly, within a mean follow-up of 107 months (range 29–214) all four patients developed HL after being treated with fludarabine. The involved sites included supra- and infradiaphragmal lymph nodes, the tonsils, as well as (in patient 4) the spleen, liver and bone marrow, and were accompanied by a generalized lymphadenopathy. All patients complained of B-symptoms and showed CLL treatment-resistant lymphadenopathies.

In two cases (patients 1 and 3), the mediastinal and retroperitoneal lymph node biopsies were infiltrated by small T lymphocytes as well as the characteristic HRSC of HL (Fig 1A). The latter were $CD30^+$ (membranous and perinuclear dot-like staining), latent membrane protein 1 of EBV $(LMP\text{-}1)^+$, $EBER^+$, $CD15^-$, $CD45RA^-$ and typically rimmed by $CD3^+$ T lymphocytes (Table III, Fig 1A,C and E). In case 2, the enlarged right tonsil (4.8 cm) showed complete architectural effacement by small lymphocytes and paraimmunoblasts, consistent with CLL and many typical EBV-infected HRSC, rimmed by $CD3^+$ T lymphocytes, 50% of the former expressing CD20 (Fig 1D). There was no evidence of sclerosis or eosinophilia. The cases were formally classified as EBV-associated mixed cellularity (MC) HL and, taking into consideration the clinical history, as an HL variant of RS.

In the fourth patient previously diagnosed with CLL, HL was first detected on the trephine bone marrow biopsy (Fig 1B) obtained for the diagnosis of persistent transfusion-dependent anaemia without evidence of haemolysis or leukopenia. HL was also manifested by B-symptoms, para-aortic lymphadenopathy and splenomegaly. Following splenectomy in spleen tissue, as well as in liver and lymph node biopsies, HL was classified as nodular sclerosis (NS) without EBV association. The HRSC remained negative for CD20 and expressed p53 in only 15%, when compared with a mean of 43% (range 30–60%) in the former cases.

Molecular findings

DNA were isolated from all analysed samples. The PCR products from the CLL and the microdissected HRSC showed similar migration patterns, indicating similar IgH rearrangements and, thus, suggestive of a clonal lymphoma relationship in patients 2 and 3, but not in patient 4 (Fig 2).

Table II. Patient characteristics.

			CLL			Hodgkin's lymphoma				
Patient	Sex	Birth date (month/year)	Diagnosis (month/year)	RAI	Therapy	Diagnosis (month/year)	Subtype, location	Stage	Therapy	Outcome
1	F	May/1922	April/1984	I	16 × COP 6 × CHOP 9 × fludarabine 22 × alemtuzumab	June/2003	Mixed cellularity, EBV-associated, retroperitoneum	1B	Irradiation	April/2004 dead with evidence of persistent CLL
2	M	March/1944	November/2000	II	6 × fludarabine/IFN-α 6 × COP	April/2004	Mixed cellularity, EBV-associated, tonsils	1B	Tonsillectomy	December/2004 alive, no evidence of Hodgkin's lymphoma, persistent CLL
3	M	January/1936	November/1997	0	6 × COP 6 × fludarabine/IFN-α 1 × IFN-α for 1 year 6 × Gupta *et al* (2002) 6 × rituximab	July/2004	Mixed cellularity, EBV-associated, mediastinum	2B	Irradiation	December/2004 dead with regressive mediastinal lymphomas, evidence of persistent CLL
4	M	August/1938	November/1994	0	2 × fludarabine	July/2004	Nodular sclerosis, no EBV association, generalized disease	4B	2 × ABVD	December/2004 alive with regressive Hodgkin's lymphoma, no evidence of CLL

COP, cyclophosphamide, oncovin, prednisolone; CHOP, cyclophosphamide, adriamycin, oncovin, prednisolone; IFN, interferon; ABVD, adriamycin, bleomycin, vinblastine, dacarbazine

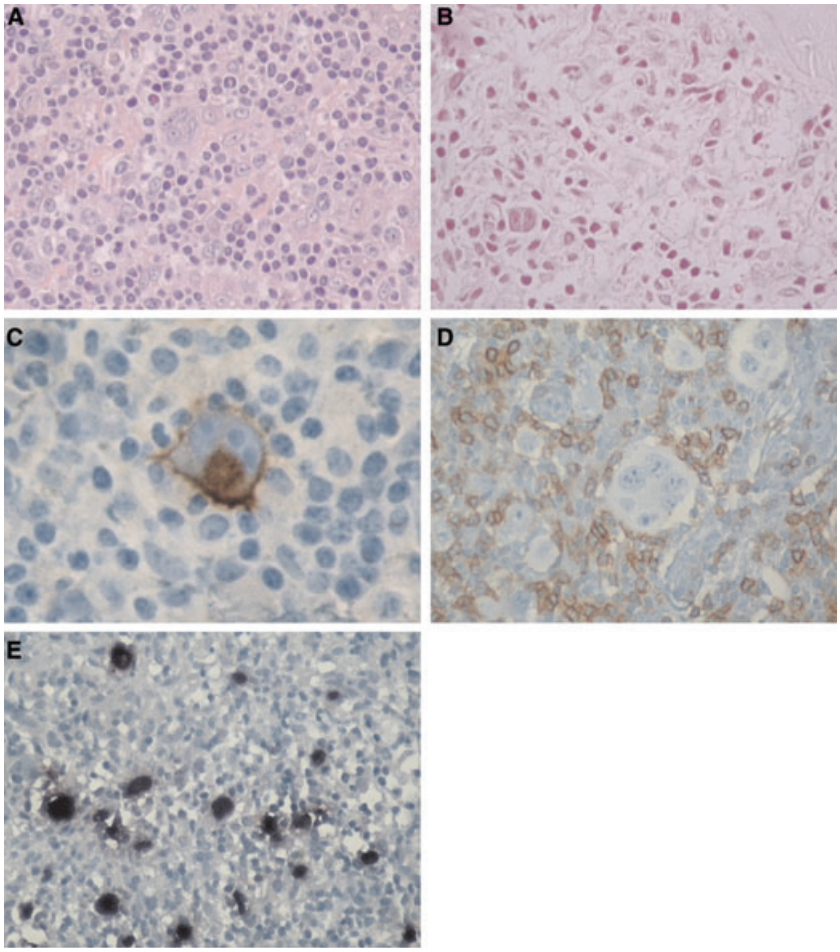

Fig 1. (A) Mediastinal mixed cellularity Hodgkin's lymphoma variant of Richter's syndrome (patient 3), H&E stain, 200×. (B) Bone marrow infiltration by nodular sclerosis Hodgkin's lymphoma (patient 4). Note the bony trabecula at the right upper corner. H&E stain, 200×. (C) CD30 expression in a Reed–Sternberg cell of Hodgkin's lymphoma variant of Richter's syndrome (patient 3), note dot-like enhancement in the Golgi region, immunoperoxidase stain, 600×; (D) T cell rimming around a Hodgkin cell in a Hodgkin's lymphoma variant of Richter's syndrome (patient 2), anti CD3-immunoperoxidase stain, 400×; (E) Epstein–Barr virus-encoded small RNA in Hodgkin cells of Hodgkin's lymphoma variant of Richter's syndrome (patient 1), Novocastra® *in situ* hybridization kit, 100×.

Table III. Immunohistochemical lymphoma characteristics.

	Immunophenotype	
Patient	Chronic lymphocytic leukaemia	Hodgkin's lymphoma
1	$CD20^+$, $CD5^+$, $CD23^+$, $CD79a^+$	$CD30^+$, $CD15^-$, LMP-1^+, $EBER^+$, CD 45^-, $CD20^+$ (10% of HRSC), $p53^+$ (40% of HRSC)
2	$CD20^+$, $CD5^+$, $CD23^+$, $CD79a^+$	$CD30^+$, $CD15^+$, LMP-$1^\pm$, $EBER^+$, CD 45^+, $CD20^+$ (50% of HRSC), $p53^+$ (30% of HRSC)
3	$CD20^+$, $CD5^+$, $CD23^+$, $CD79a^+$	$CD30^+$, $CD15^-$, LMP-1^+, $EBER^+$, CD 45^-, $CD20^-$, $p53^+$ (60% of HRSC)
4	$CD20^+$, $CD5^\pm$, $CD23^\pm$, $CD79a^+$	$CD30^+$, $CD15^+$, LMP-1^-, $EBER^-$, CD 45^+, $CD20^-$, $p53^+$ (15% of HRSC)

Discussion

Richter's syndrome is well known for its interruption of the indolent course of CLL in about 2–5% of patients, by transformation to a more aggressive terminal disease (Keating *et al*, 1998). The high-grade component usually consists of monomorphic large B-cells, classified as DLBCL with fatal outcome and a median survival of <6 months. Transformation of CLL to HL, also called HL variant of RS, is considerably less common and is determined by the occurrence of areas of classical HL with a distinct HRSC immunophenotype ($CD30^+$, $CD15^\pm$, $CD45RA^-$, $CD20^\pm$, LMP-$1^\pm$) (Williams *et al*, 1991; Momose *et al*, 1992; Shin *et al*, 1993; Pescarmona *et al*, 2000). It should be distinguished from a CLL variant with scattered HRS-like cells ($CD30^+$, $CD15^-$, $CD45RA^+$, $CD20^+$, $LMP^\pm$) for therapeutic purposes (Choi & Keller, 1981; Brecher & Banks, 1990; Weisenberg *et al*, 1995), as the latter does not represent disease transformation. The HRSC in our cases were $CD30^+$, $CD15^\pm$, $CD45RA^-$, LMP-$1^\pm$ and variably positive for CD20. In classical HL, HRSC express the B-cell marker CD20 in about

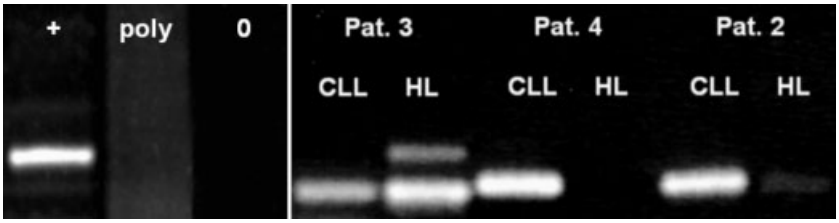

Fig 2. Polynat gel electrophoresis of FR-III/JH IgH PCR products (lanes are numbered left to right). Lane 1: monoclonal positive control; lane 2: polyclonal control; lane 3: negative control. In each case (lanes 4–9), the DNA from a whole bone marrow section harbouring CLL was paired with the DNA from the corresponding microdissected Hodgkin and Reed–Sternberg cells (HRSC) and then analysed. In two cases [patient (pat.) 3, lanes 4/5 and pat. 2, lanes 8/9] a similar pattern of migration of the monoclonal IgH products is suggestive of the presence of clonally related lymphomas.

20% of cases, which appears to be of prognostic significance (Tzankov *et al*, 2003). It has been shown that rituximab (Rehwald *et al*, 2003) may be useful for relapse therapy in such cases. Our observations of HL occurring in CLL, however, do not indicate high sensitivity of secondary HL to this monoclonal antibody since CD20 is inconsistently expressed.

Adiga *et al* (2003) recently reviewed the literature for CLL transformation to HL and found 88 case reports from 1975 to 2002 with a maximum of eight cases reported in one series (Brecher & Banks, 1990; Weisenberg *et al*, 1995). The evidence reported so far indicates that some HL variant of RS in CLL could be associated with fludarabine pretreatment (Rubin *et al*, 1994; Shields *et al*, 1997; Adiga *et al*, 2003; de Leval *et al*, 2004), whereas other cases were not (Brecher & Banks, 1990; Williams *et al*, 1991; Kanzler *et al*, 2000). The frequency of patients with CLL transforming to HL is about 0.5% (Fayad *et al*, 1996; Mauro *et al*, 1999; Pescarmona *et al*, 2000). However, in a long-term follow-up report, four cases (2.3%) of HL were found among 174 CLL patients treated with fludarabine (Keating *et al*, 1998). The interval between diagnosis of CLL and subsequent development of HL ranged from 0 to 212 months, with a median of 61.8 months (in our study, 107). Notably, all four of our patients developed HL variant of RS within a mean period of 24 months (range 4–38) after treatment with fludarabine got started. No cases of secondary HL following CLL were reported at our institutions between 1993 and 2000, prior to the approval of fludarabine for treatment of CLL. Rubin *et al* (1994) first suggested a possible causal relationship between fludarabine therapy and the occurrence of EBV-associated HL. The nucleoside analogue fludarabine is highly effective in treating CLL, but can cause severe T-cell lymphopenia as a side-effect. This drug-induced T-cell immunodeficiency in combination with the intrinsic T-cell dysfunction (Scrivener *et al*, 2003) in CLL patients may increase the risk of EBV-associated B-cell lymphoproliferative disorders (Shields *et al*, 1997; Abruzzo *et al*, 2002). Importantly, EBV can be detected in about 50% of HL variant of RS, which is more frequent than in European sporadic HL cases (Krugmann *et al*, 2003). It is well known that the amount of latently EBV-infected cells can increase during immunosuppression and fludarabine treatment (Lazzarino *et al*, 1999) appears to further enhance the CLL-related immunodeficiency.

The clonal relationship between CLL cells and HRSC has been investigated in several studies (Rubin *et al*, 1994; Ohno *et al*, 1998; Kanzler *et al*, 2000; Pescarmona *et al*, 2000; van den Berg *et al*, 2002). de Leval *et al* (2004) described two additional cases following fludarabine treatment and reviewed the literature for cases with molecular analysis of clonal relationship between CLL and HL. In nine such cases, the clonal relationship was ultimately established, five being of the same (three EBV^-; two with unknown EBV status) and four of distinct (all EBV^+) clonal origin. In our series, HRSC in two of three EBV-associated MC HL were presumably clonally related to CLL, while in the fourth case (NS HL lacking EBV infection), no evidence for a clonal relationship was found. p53 was expressed in a mean of 43% of HRSC with MC HL, but in 15% with NS. Taking into consideration the clinical presentation (the NS HL patient was classified as stage IV), EBV status and the expression of p53, we assume distinct pathogenic pathways for the MC and NS HL in our CLL patients: the first three being EBV-induced, clonally related to CLL and expressing p53 in about half of the HRSC, while the HL of the last patient was neither associated with viral infection nor clonally related to CLL, suggesting a sporadic event rather than NHL evolution.

In conclusion, HL can occur in CLL patients at any site and up to 17 years after the initial diagnosis, especially after treatment with fludarabine. The development of secondary HL might be triggered by an EBV infection. In our cases, HL presented with B-symptoms and CLL treatment-resistant lymphadenopathy. Most cases appeared to be of the MC type and clonally related to CLL. In addition, isolated cases of HL without a clonal relationship to the pre-existing CLL, of the NS type that are EBV-negative may also occur. Importantly, as the majority of HL variants of RS seem to be related to or triggered by EBV infection, monitoring of the viral load during fludarabine treatment might be useful for preventing CLL transformation to an EBV-associated HL, and to prove this, large randomized prospective studies are needed.

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