Three-dimensional Telomere Signatures of Hodgkin- and Reed-Sternberg Cells at Diagnosis Identify Patients with Poor Response to Conventional Chemotherapy

Abstract

In classic Hodgkin lymphoma (HL) the malignant mononuclear Hodgkin (H) and multinuclear Reed-Sternberg (RS) cells are characterized by a distinct three-dimensional nuclear telomere organization with shortening of the telomere length and the formation of telomeric aggregates. We asked if the severity of these telomere changes correlates with the clinical behavior of the disease. We retrospectively evaluated three-dimensional telomere organization by quantitative fluorescent in situ hybridization (Q-FISH) of diagnostic biopsies from 16 patients who were good responders and compared them with 16 diagnostic biopsies of 10 patients with refractory or relapsing HL (eight initial biopsies, four confirming progressions, and four confirming relapses). The H cells from patients with refractory/relapsing disease contained a significantly higher percentage of very small telomeres ($P = .027$) and telomere aggregates ($P = .032$) compared with H cells of patients entering rapid remission. These differences were even more significant ($P = .002$ and $P = .013$, respectively) when comparing the eight initial diagnostic biopsies of refractory/relapsing HL with diagnostic biopsies of eight patients with ongoing long-lasting remission (mean of 47 months). This specific three-dimensional telomere Q-FISH signature identifies these highly aggressive mononuclear H cells at the first diagnostic biopsy and thus may offer a new molecular marker to optimize initial treatment.

Introduction

Telomeres are the nucleoprotein complexes at the ends of chromosomes. Telomeric DNA consists of multiple double-stranded TTAGGG repeats and ends in a single-stranded overhang of the G-rich 3′ strand [1]. Furthermore, a number of specific proteins, either binding telomeric DNA directly or being associated with telomeric chromatin, called shelterin complex, are found on telomeres [2,3]. Many cancer cells display chromosomal aberrations that are the direct result of telomere dysfunction [4,5] and the three-dimensional organization of telomeres is altered in cancer cells [6,7].

This basic finding led to an advanced understanding of genetic changes in early cancer cells and proved that telomere organization is key to genome stability versus instability [8,9]. We have shown that each nucleus has a specific three-dimensional telomeric signature that defines it as normal or aberrant. Four criteria define this difference: 1) nuclear telomere distribution, 2) the presence/absence of...
Materials and Methods

The binuclear or multinuclear Reed-Sternberg cells (RS cells), the diagnostic element of Hodgkin lymphoma (HL), derive from mononuclear precursors called Hodgkin (H) cells, through endoreduplication and have a limited capacity to further divide [12–14]. H cells originate from germinal center B cells [15], and small circulating clonotypic B cells, putative precursors of H cells, have been identified by flow cytometry [16]. H and RS cells show high telomerase activity [17,18] and express abundant telomerase RNA [19].

Using a three-dimensional quantitative fluorescent in situ hybridization (Q-FISH) technique to visualize telomeres in cultured cells and biopsies [8], we recently characterized the transition from mononuclear H to multinuclear RS cells at the molecular level [20–22]. We demonstrated that RS cells are true end-stage tumor cells in both classic Epstein-Barr virus (EBV)–negative and EBV-positive HL. The number of nuclei in these RS cells correlates closely with the three-dimensional organization of telomeres and further nuclear divisions are hampered by sustained telomere shortening or loss and telomere aggregation. The increase in very small telomeres and aggregates in these RS cells compared with their mononuclear precursor H cells is highly significant (P < .01). Such RS cells contain telomere/DNA–poor “ghost” nuclei and giant “zebra” chromosomes including up to seven different chromosomal partners as revealed by spectral karyotyping (SKY). These molecular changes are the result of multiple breakage-bridge-fusion (BBF) cycles [22].

With the ability of three-dimensional nuclear telomere analysis to be performed on paraffin-embedded tissue blocks to specifically address the nuclear structure in the minor population of cells in HL that are the malignant component (H and RS cells), we had the tool to now ask whether there were differences in the H and RS cells of patients with primary refractory or relapsing HL compared with those of good responders. In this study, we analyzed 16 diagnostic lymph node biopsies from patients entering rapid remission and compared them to 16 lymph node biopsies from 10 patients who went on to have relapse or refractory disease. Our results show significant differences in three-dimensional telomere dynamics of relapsing/progressing disease.

Study Design

From January 2006 to January 2011, patients diagnosed by lymph node biopsy for HL or closely related diseases at the Université de Sherbrooke were enrolled in the trial to evaluate telomere structure by three-dimensional telomere Q-FISH analysis performed on archived histology slides of diagnostic biopsies. This tumor biology study did not affect patient management. Forty-two patients entered the study (most of them prospectively), 33 had histologic diagnosis (lymph node) of classic HL, 4 had lymphocyte-predominant nodular HL, 3 had EBV-associated angioimmunoblastic T-cell lymphoma (LMP1+), 1 had T-cell anaplastic large cell lymphoma ALK+, and 1 had EBV-positive large B-cell lymphoma. Among the patients with HL, four were referred from other centers (three with relapsing/progressing disease).

Clinical Response Definition

Remission from HL was defined by fluorodeoxyglucose (FDG) positron emission tomography–computed tomography (PET/CT) scan (and bone marrow biopsy for patients with initial bone marrow involvement). The activity of involved sites had to disappear completely by follow-up FDG PET/CT scan when compared with the FDG PET/CT scan performed at diagnostic workup. In patients with initial bone marrow involvement, a follow-up bone marrow biopsy had to get cleared of CD30+ tumor cells. Follow-up FDG PET/CT scan was performed at the treating physician’s discretion but at least after the fourth cycle of chemotherapy. Patients in remission had at least two additional FDG PET/CT scans within the first year after completion of treatment.

Ethics

The study design has been approved by the institutional ethics committee of the Université de Sherbrooke and the institutional review board of the University of Manitoba. Informed consent of each patient has been obtained, and the investigations have been conducted according to the Declaration of Helsinki.

Tissue Slides

Serial sections (5 μm thick) from archived formalin-fixed paraffin-embedded tissue from every lymph node biopsy diagnostic for classic Hodgkin’s disease (initial diagnosis, diagnosis of relapse, diagnosis of progression) were deparaffinized two times for 15 minutes at room temperature in xylene and rehydrated in reverse-graded series of ethanol (incubations are 100%, 75%, 70%, and 50% for 10 minutes each). The slides were then dipped briefly in ddH2O and then transferred into phosphate-buffered saline and used for hematoxylin-eosin staining (serial section no. 1), immunostaining (anti-CD30, serial section no. 2; anti-LMP1, serial section no. 3), and Q-FISH (serial section no. 4).

Identification of LMP1 Expressing H and RS Cells on Serial Sections with Combined Three-dimensional DAPCyt3 Telomere Q-FISH Nuclear Staining

On serial section no. 3, lymph node regions with LMP1-expressing H and RS cells were identified at a magnification of 200× and individual cells were further confirmed at a magnification of 630×. Subsequently, on serial section no. 4, the corresponding region was identified at a magnification of 200×, and the corresponding H and RS cells (hence, LMP1-expressing) were further analyzed for three-dimensional nuclear telomere organization at a magnification of 630×.

Immunohistochemistry

Immunostaining was performed by standard indirect immunoperoxidase technique using primary monoclonal mouse antibodies anti-CD30 (Ber-H2) and anti-LMP1 (clones CS1-CS4) from DAKO (Glostrup, Denmark) at a dilution of 1:40 and 1:50, respectively. Photomicrographs were performed by using a Zeiss Axioskop 2 microscope (Carl Zeiss Canada, Toronto, Canada) with a Polaroid C11806TV camera and Polaroid DMC2 v2.01 software (Kodak, Toronto, Canada).

Telomere Q-FISH

The telomere FISH protocol was performed [6,8] by using Cy3-labeled peptide nucleic acid probes (DAKO). Imaging of interphases after telomere FISH was performed by using Zeiss AxioImager Z1 with a cooled AxioCam HR B&W, DAPI, Cy3 filters in combination with a Planapo 63×/1.4 oil objective lens. Images were acquired by...
using AxioVision 4.6 and 4.8 (Zeiss) in multichannel mode followed by constraint iterative deconvolution as specified in the next paragraphs.

Three-dimensional Image Acquisition

At least 30 H-cell interphase nuclei and 30 RS-cell interphase polycaria were analyzed in each lymph node slide. AxioVision 4.6 and 4.8 with deconvolution module and rendering module were used. For every fluorochrome, the three-dimensional image consists of a stack of 40 images with a sampling distance of 200 nm along the $z$ and 107 nm in the $x$ and $y$ direction. The constraint iterative algorithm option was used for deconvolution [23].

Three-dimensional Image Analysis for Telomeres

Telomere measurements were done with TeloView [6,24]. By choosing a simple threshold for the telomeres, a binary image is found. On the basis of this threshold, the center of gravity of intensities is calculated for every object, resulting in a set of coordinates $(x, y, z)$ denoted by crosses on the screen. The integrated intensity of each telomere is calculated because it is proportional to the telomere size [25].

a) Segmental nuclear volume: Nuclear volume within one 5-μm-thin nuclear section of H cells or RS cells is calculated according to the three-dimensional nuclear DAPI staining as previously described [26]. Contrary to whole cell preparations (cells or cell lines), where the nuclei can be visualized with their entire volumes and z-stack analysis along the $z$ direction over 15 μm allows to calculate the entire nuclear volume, in tissue sections, analysis of the nuclear volume is limited to 5-μm nuclear segments (as used as a standard for histopathologic diagnosis) along the $z$ direction. Deparaffinized tissue slides of 10 and 15 μm thickness are technically unsatisfactory for Q-FISH analysis. Thus, the segmental nuclear volume represents approximately 30% to 50% of the total nuclear volume of H cells (with a nuclear diameter of approximately 10-15 μm) and approximately 15% to 25% of the total nuclear volume of RS cells (diameter of two up to several nuclei measures approximately 20-40 μm).

b) Segmental telomere number: Segmental telomere number is the sum of all very small, small, midsized, and large telomeres and aggregates identified within one 5-μm-thin nuclear section of an H cell or RS cell.

c) Segmental telomere intensity: Segmental telomere intensity is the sum of intensities of all very small, small, midsized, and large telomeres and aggregates identified within one 5-μm-thin nuclear section of an H cell or RS cell (viz., $\Sigma_{2 \times 15,000 \text{ units}} > \Sigma_{7 \times 4000 \text{ units}}$).

d) Mean telomere intensity: Mean telomere relative fluorescent intensity (size) of all telomeres within a given segmental volume.

e) Telomere size: Telomeres with a relative fluorescent intensity ($x$ axis) ranging from 0 to 5000 units are classified as very small, with an intensity ranging from 5000 to 15,000 units as small, with an intensity from 15,000 to 30,000 units as midsized, and with an intensity greater than 30,000 units as large [21].

f) Telomere aggregates: Telomere aggregates are defined as clusters of telomeres that are found in close association and cannot be further resolved as separate entities at an optical resolution limit of 200 nm [9].

Statistical Analysis

In each case of classic HL, at least 30 H cells and 30 RS cells have to be analyzed by three-dimensional telomere Q-FISH. If there were fewer than 30 H and RS cells present on a diagnostic slide, it was insufficient for reliable statistical analysis, and the slide was not included. Unequivocal statistical analysis could be performed on 32 diagnostic biopsies of 26 patients with diagnosis of classic HL (in patients with relapsing/progressing disease, second and third biopsies diagnostic for relapse or progression were analyzed; Table 1B). For each case, normally distributed parameters are compared between the two types of cells using nested analysis of variance or two-way analysis of variance. Multiple comparisons using the least square means tests followed where interaction effects between two factors were found to be significant. Other parameters that were not normally distributed were compared using a nonparametric Wilcoxon rank sum test. Significance level were set at $P = .05$. Analyses were done using SAS v9.1 programs (SAS Institute Inc, Cary, NC).

Results

Clinical Data Including Outcome

A total of 32 diagnostic biopsies were adequate for statistical analysis; 16 biopsies from 16 patients belonged to patients entering rapid remission, belong to group A (remission documented after fourth cycle of chemotherapy at the latest), and 16 diagnostic biopsies (eight initial biopsies including two patients never entering remission, four documenting relapse and four disease progression) from 10 patients belonged to group B. The clinical data including treatment modalities, international prognostic score (IPS), and outcome of both groups are shown on Table 1, A and B, respectively. The groups were similar for age and IPS score. Stage IV disease was more frequent in patients of group B ($3 \text{ vs } 1$).

Three-dimensional Telomere Q-FISH Group A

The nuclear telomere organization of patients entering rapid remission is shown on Table 2A. Compared with mononuclear H-cell nuclei, RS-cell nuclei have a significantly larger volume and are characterized by a significant increase in the number of very small telomeres and aggregates ($P < .01$), as defined in Materials and Methods, whereas the total telomere mass (segmental telomere intensity) was nearly unchanged ($P = .395$). As a result, the mean telomere intensity in RS cells is much lower compared with that in H cells ($P < .01$). When the total telomere mass is normalized to a virtual nuclear volume of 1000 μm$^3$, RS cells show a significant decrease in the total telomere mass ($P < .01$) compared with H cells underscoring the shortening and loss of telomeres associated with the transition from H to RS cells, confirming our earlier observations [20,21].

Three-dimensional Telomere Q-FISH Group B

The nuclear telomere organization in patients with progressing or relapsing disease is shown on Table 2B. Again, compared with mononuclear H-cell nuclei, RS-cell nuclei have a significantly larger volume and are characterized by a significant increase in number of very small telomeres and aggregates ($P < .01$) but also by a significant increase ($P < .01$) of the total telomere mass (segmental telomere intensity). However, when the total telomere mass is normalized to a virtual nuclear volume of 1000 μm$^3$, this increase in the total telomere mass is no longer identifiable ($P = .493$), with a remaining still-significant
decrease of the mean telomere intensity ($P = .014$). Most importantly, these differences between RS and H cells—although analogous to those identified within the rapid remission group A—do occur at a much advanced level. For instance, the number of very small telomere increases between H and RS cells from 48.0\% to 72.4\%, is identified in group A (Table 2), whereas a similar increase, but at a much lower level, from 391 vs 666 m^3\,), whereas the number of aggregates per cell (3.1 vs 3.4) and the percentage of very small telomere (68.8\% vs 72.4\%) are very similar; however, both, aggregates and a very small telomere of H cells of group B, have significantly increased compared to the eight initial biopsies of refractory/relapsing HL (biopsies 1-7.12) entering rapid and still capable of completing mitosis resulting in two mono- nuclear daughter H cells, have already telomere characteristics identified in RS cells of the rapid remission cases as shown in the right track of Table 3. These H cells of group B differ from the RS cells of group A in terms of the expected difference in nuclear volume (391 vs 666 m^3\,), whereas the number of aggregates per cell (3.1 vs 3.4) and the percentage of very small telomere (68.8\% vs 72.4\%) are very similar; however, both, aggregates and a very small telomere of H cells of group B, have significantly increased compared with those of H cells of group A ($P = .032$ and $P = .027$, respectively; first track of Table 3). These differences are even more significant for very small telomeres ($P = .002$) and for aggregates ($P = .013$) when comparing the eight initial biopsies of refractory/relapsing HL (biopsies 17a, 19a, 20a, 21a, 22a, 23a, 25a, and 26a) with the diagnostic biopsies eight patients (biopsies 1-7.12) entering rapid and still ongoing sustained remission (27-70 months; mean of 47 months). Notably, these three-dimensional telomere dynamics were identified before identical ABVD chemotherapy (except case 23a) in both groups and, consequently, the different clinical course independent of initial treatment modality. For the H cells of the refractory/relapsing cases, this is consistent with multiple completed rounds of cellular division (i.e., repeated telomere shortening) that are

Comparison of the Three-dimensional Telomere Characteristics of Groups A and B

Most importantly, the mononuclear H cells of progressing/relapsing cases, still capable of completing mitosis resulting in two mono- nuclear daughter H cells, have already telomere characteristics identified in RS cells of the rapid remission cases as shown in the right track of Table 3. These H cells of group B differ from the RS cells of group A in terms of the expected difference in nuclear volume (391 vs 666 m^3\,), whereas the number of aggregates per cell (3.1 vs 3.4) and the percentage of very small telomere (68.8\% vs 72.4\%) are very similar; however, both, aggregates and a very small telomere of H cells of group B, have significantly increased compared with those of H cells of group A ($P = .032$ and $P = .027$, respectively; first track of Table 3). These differences are even more significant for very small telomeres ($P = .002$) and for aggregates ($P = .013$) when comparing the eight initial biopsies of refractory/relapsing HL (biopsies 17a, 19a, 20a, 21a, 22a, 23a, 25a, and 26a) with the diagnostic biopsies eight patients (biopsies 1-7.12) entering rapid and still ongoing sustained remission (27-70 months; mean of 47 months). Notably, these three-dimensional telomere dynamics were identified before identical ABVD chemotherapy (except case 23a) in both groups and, consequently, the different clinical course independent of initial treatment modality. For the H cells of the refractory/relapsing cases, this is consistent with multiple completed rounds of cellular division (i.e., repeated telomere shortening) that are

ABVD indicates adriamycin, bleomycin, vinblastine, dacarbazine; BEAM, carmustine, etoposide, cytarabine, cyclophosphamide; BEAC, carmustine, etoposide, cytarabine, melphalan; CVPP-AO, cyclophosphamide, vinblastine, procarbazine, prednisone/adriamycin, oncovin; DBVE-PC, doxorubicin, bleomycin, vinblastine, etoposide/prednisone, cyclophosphamide; ESHAP, etoposide, cytarabine, cyclophosphamide, melphalan; ICE, ifosphamide, carboplatin, etoposide; IFRT, involved field radiation therapy; IPS, international prognostic score; MC, mixed cellularity classic HL; NS, nodular sclerosis classic HL; R-Gem, rituxan, gemcitabine; **Biopsies with only RS cells statistically analyzed because the number of H cells in these biopsies was fewer than 30.

*Comparison of the Three-dimensional Telomere Characteristics of Groups A and B

Most importantly, the mononuclear H cells of progressing/relapsing cases, still capable of completing mitosis resulting in two mono- nuclear daughter H cells, have already telomere characteristics identified in RS cells of the rapid remission cases as shown in the right track of Table 3. These H cells of group B differ from the RS cells of group A in terms of the expected difference in nuclear volume (391 vs 666 m^3\,), whereas the number of aggregates per cell (3.1 vs 3.4) and the percentage of very small telomere (68.8\% vs 72.4\%) are very similar; however, both, aggregates and a very small telomere of H cells of group B, have significantly increased compared with those of H cells of group A ($P = .032$ and $P = .027$, respectively; first track of Table 3). These differences are even more significant for very small telomeres ($P = .002$) and for aggregates ($P = .013$) when comparing the eight initial biopsies of refractory/relapsing HL (biopsies 17a, 19a, 20a, 21a, 22a, 23a, 25a, and 26a) with the diagnostic biopsies eight patients (biopsies 1-7.12) entering rapid and still ongoing sustained remission (27-70 months; mean of 47 months). Notably, these three-dimensional telomere dynamics were identified before identical ABVD chemotherapy (except case 23a) in both groups and, consequently, the different clinical course independent of initial treatment modality. For the H cells of the refractory/relapsing cases, this is consistent with multiple completed rounds of cellular division (i.e., repeated telomere shortening) that are
completed with intact nuclear and cytoplasm segregation indicating primary aggressive disease. This telomere profile contrasts with that of rapid remission cases (Figure 3, A and B). Importantly, the mean segmental telomere intensity (mean total telomere mass) is unchanged between H cells of group B (162,215 units) and RS cells of group A (167,241 units) as well as the mean segmental telomere number (31.0 vs 32.3). Thus, the mononuclear H cells of refractory/relapsing cases behave in their three-dimensional nuclear telomere signature like end-stage RS cells of the remission group A without being it (Figure 4).

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**Figure 1.** Three-dimensional nuclear organization of telomeres (red) and total nuclear DNA (blue) in RS cells of relapsing HL. Trinuclear RS cell (biopsy 20b) showing unequal nuclear distribution of mainly very small telomere when compared with surrounding lymphocytes, which contain midsized and large telomere. Small and very small telomere are clustered in the upper nucleus (1) of the RS cell whereas the left (2) and lower right nucleus (3) behave like “ghost” elements with only few very small telomere. Inset highlights clustering and very small telomere in the RS cell compared with adjacent lymphocytes.

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**Table 2.** Three-dimensional Telomere Characteristics of RS Cells and H Cells of 16 (A; Rapid Remission Group A) and 14 Diagnostic Biopsies (B; Progression/Relapse Group B).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RS cells (n = 510; 223 LMP1+)</th>
<th>H cells (n = 524; 235 LMP1+)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segmental nuclear volume</td>
<td>666 μm³ (422)*</td>
<td>366 μm³ (201)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Segmental telomere number</td>
<td>52.3 (23.1)</td>
<td>20.9 (15.8)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Segmental telomere intensity</td>
<td>162,215 units (148,233)</td>
<td>154,824 units (182,473)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean telomere intensity</td>
<td>5425 units (3362)</td>
<td>7682 units (4496)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Very small telomere (0-5000 U)</td>
<td>72.4% (15.2)</td>
<td>48.0% (18.4)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Segmental telomere aggregates</td>
<td>3.4 (3.3)</td>
<td>1.9 (2.3)</td>
<td>&lt;.01</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>H cells (n = 473; 72 LMP1+)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Segmental nuclear volume</td>
<td>718 μm³ (446)*</td>
<td>391 μm³ (193)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Segmental telomere number</td>
<td>44.0 (33.3)</td>
<td>31.0 (21.7)</td>
<td>&lt;.01</td>
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<tr>
<td>Segmental telomere intensity</td>
<td>197,951 units (175,103)</td>
<td>167,241 units (125,141)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Mean telomere intensity</td>
<td>5159 units (6968)</td>
<td>5986 units (3263)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Very small telomere (0-5000 U)</td>
<td>81.8% (15.5)</td>
<td>68.8% (15.1)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Segmental telomere aggregates</td>
<td>4.9 (4.7)</td>
<td>3.1 (3.2)</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

*Standard deviation.
In summary, we conclude that H cells of relapsing/refractory HL differ significantly from their counterparts of cases entering rapid and persistent remission. A hallmark is the very high percentage of very small telomeres and aggregates in these H cells.

**Discussion**

Over the past years, the success rate of modern treatment modalities for HL has stagnated at approximately 80% to 85% [27]. In approxmately 20% of patients, the disease still progresses or relapses, despite...
multiple chemotherapies, radiation therapy, and autologous bone marrow transplantation, demonstrating the need for a better molecular knowledge of the biology of the malignant cells, mononuclear H, and multinuclear RS cells [28,29]. It is most important to look for novel treatment approaches for this group of patients [30] and to identify such patients already at diagnosis before any therapy. Beside a high IPS of advanced-stage disease [31], microenvironment factors such as fibroblast activation and extracellular matrix remodeling [32], high

Figure 3. Typical telomere profiles associated with refractory disease (A) and rapid remission (B). Telomere distribution according to size in mononuclear H (blue) and at least binuclear RS cells (red). Results are based on three-dimensional analysis of at least 30 H and 30 RS cells in each biopsy. Frequency (y axis) and relative fluorescent intensity, that is, the size of telomeres (x axis) in a diagnostic 5 μm lymph node section. (A) Female patient, 77 years old, nodular sclerosis subtype, LMP1-expressing stage IIIIB, IPS 4, refractory disease, death after 11 months (case 17). In both, H and RS cells, there is a highly significant shift ($P < .01$) from midsized and small telomeres to very small telomeres including “t-stumps” of 0 to 5000 relative fluorescence units. Importantly, already the mononuclear H cells exhibit a three-dimensional telomere profile usually identified only in RS cells (arrow). (B) Male patient, 71 years old, mixed cellularity subtype, LMP1-expressing stage IIIIB, IPS 4, in remission for 62 months, documented after 4 × ABVD (case 12). In RS cells, the highly significant shift to very small telomeres is still observed, whereas it is completely absent in H cells (arrow). Thus, H cells in aggressive disease do, contrary to H cells in cases entering persistent remission, already contain multiple very small telomeres consistent with having passed multiple rounds of mitosis without ending up in multinucleated end-stage RS cells. Notably, this is observed in cases with nearly identical clinical presentation.
and more aggregates (stage HL are the Hodgkin cell lines L-428, HDLM-2, L-1236, and although having passed through multiple rounds of mitotic division, do contain much higher numbers of very small telomeres (these differences are even more pronounced; H cells of aggressive HL mission (mean of 47 months) before identical ABVD chemotherapy, biopsies of eight patients entering rapid and still ongoing long re-

Figure 4. Telomere distribution according to size in H and RS cells in rapid remission and relapsing/refractory HL. The mean telomere distributions (±SEM) in H and RS cells of 16 patient biopsies associated with rapid remission (group A) and 16 biopsies (8 initial biopsies, 4 relapses, and 4 progressions) from 10 patients (group B) are shown. Results are based on three-dimensional analysis of at least 30 H and 30 RS cells in a diagnostic 5-μm-thin lymph node section of each biopsy. Frequency (y axis) and relative fluorescent intensity, that is, the size of telomeres (x axis) are shown. There is a highly significant shift (P < .01) from small telomere (>5000 relative fluorescence units) to very small telomere including “t-stumps” of 0 to 5000 relative fluorescence units in all four curves. However, mononuclear H cells of aggressive disease have nearly as much very small telomere (P = .374) as RS cells of cases entering rapid and lasting remission indicating multiple rounds of mitotic division without progression to end-stage RS cells.

number of macrophages [33], and gains of chromosome 16p11.2-13.3 [34] are associated with unfavorable prognosis. On the other hand, the expression of CD20 in H and RS cells [35] and the negative FDG PET/CT scan results after two cycles of chemotherapy [36] are associated with a better prognosis. However, currently, there has not been a cytologic/morphologic means to discern aggressive H and RS cells from their chemotherapy-sensitive counterparts.

Here we show highly significant differences in the three-dimensional telomere characteristics between H and RS cells of patients entering rapid and sustained remission on first-line chemotherapy and those of patients who have a relapsing or refractory disease course (Tables 2, A and B). H cells of refractory/relapsing cases already have a three-dimensional telomere signature corresponding to that one of RS cells in the remission group (Table 3 and Figure 4) without being end-stage tumor cells. Importantly, when comparing the eight initial diagnostic biopsies of the refractory/relapsing group with diagnostic biopsies of eight patients entering rapid and still ongoing long remission (mean of 47 months) before identical ABVD chemotherapy, these differences are even more pronounced; H cells of aggressive HL do contain much higher numbers of very small telomeres (P = .002) and more aggregates (P = .013) than do H cells of diagnostic biopsies entering long-lasting remission. H cells of refractory/relapsing cases—although having passed through multiple rounds of mitotic division, as shown by accumulation of very small telomeres and increase in aggregates—are still capable to of further cell division and thus will increase the tumor bulk.

Modeling the telomere abnormalities found in relapsing/refractory stage HL are the Hodgkin cell lines L-428, HDLM-2, L-1236, and U-HO1, all derived from advanced-stage HL, three of them refractory, where by far most cells (>90%) are mononuclear H cells [37–40]. These mononuclear H cells, despite having a high complexity of chromosomal aberrations, are able to undergo mitotic division. Their transition to multinuclear end-stage RS cells is associated with a further increase in chromosomal complexity [22], formation of multiple aberrant mitotic spindles, telomere aggregates, and a further shortening of telomere [20,41]. Such extremely small telomere, also called t-stumps [42], and telomere aggregates are hallmarks of cancer cells [10].

Indeed, using innovative three-dimensional quantitative FISH techniques for telomere dynamics and whole chromosome painting, as well as spectral karyotyping (SKY), we have gained detailed molecular insights into the transition of mononuclear H- to multinuclear RS cells [20–22]. We identified RS cells of classic HL as true end-stage tumor cells, characterized by abundant very small telomeres, giant “zebra” chromosomes containing concatameric repeats from two different, or parts originating from up to seven different chromosomes, and “ghost” nuclei poor or void of telomeres and chromosomes, consistent with the hypothesis of multiple ongoing BBF cycles at the origin of RS-cell generation. In line with this observation, three-dimensional super resolution microscopy identified internuclear DNA bridges between RS-cell nuclei and thus corroborated the generation of RS cells as a result of multiple BBF cycles [22]. Thus, in refractory/relapsing HL, the H cells have undergone already at diagnosis multiple BBF cycles still allowing proper chromosomal segregation.

Our pathogenetic model is further supported by the emergence of HL relapses with unrelated clonal rearrangements, as recently reported [43]. This dynamic process of nuclear remodeling probably begins in the few clonotypic circulating B cells identified recently by Jones et al. in HL [16] or, in EBV-associated HL, even earlier in activated EBV-infected tonsillar germinal center B cells that escape immunosurveillance [44]. Indeed, telomere shortening and chromosomal instability in peripheral blood lymphocytes of HL patients before any treatment have been identified [45].

We have been able to identify a novel prognostic tool predictive of poor biologic activity in HL—the three-dimensional nuclear telomere structure. Studies are ongoing to confirm this observation in an independent cohort of HL patients and to study a large-enough cohort to evaluate whether this is an independent biomarker once stage, subtype, age, and other known prognostic factors are analyzed. The ability to study the nuclear structure of the malignant subpopulation of HL has the potential of providing a major advance to the field of HL management.

In summary, H and RS cells are characterized by a distinct three-dimensional telomere nuclear organization. Three-dimensional telomere signatures of mononuclear H cells in refractory/relapsing HL are significantly different from those in HL rapidly entering sustained remission. In refractory Hodgkin disease, the H cells show a three-dimensional telomere fingerprint (significantly higher number of very small telomeres and aggregates) nearly identical to that one of RS cells of the remission group. These H cells appear to escape standard chemotherapy but may be responding to novel therapeutic approaches [30].

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References


