Genomic imbalances associated with secondary acute leukemias in Hodgkin lymphoma

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Abstract. Secondary tumors and leukemias are major complications in Hodgkin lymphoma (HL). They likely arise from clonal selection of cells that have accumulated genomic lesions induced by chemo- and radiotherapy and may be further promoted by the loss of DNA repair and/or other pathways ensuring the fidelity of replicated DNA. To distinguish genomic imbalances associated with the development of acute myeloid leukemia (AML) in HL we used an array-based comparative genomic hybridization (aCGH) strategy on whole lymph node biopsies of HL patient. Genomic imbalances (amplifications and deletions) associated with AML outcome in 3 classic HL patients, at clinical diagnosis they exhibited a discrete individual variability. Three amplifications and 5 deletions were shared by all 3 patients. They involved AFM137XA11, a 9p11.2 pericentric region; FGFR1, the FGF receptor most frequently translocated in AML; PPARBP, a co-activator of nuclear receptors RARα, RXR and TRβ1; AFM217YD10, a 17q25 telomeric region; FGR, an SRC2 kinase involved in cytokine production by NK and CD4+ NKT cells; GATA3, a Th2-specific transcription factor; TOP1, involved in DNA recombination and repair; WT1, a transcription factor involved in CD8+ T cell response against leukaemic blasts. Immunohistochemistry confirmed aCGH results and distinguished the distribution of either amplified or deleted gene products in neoplastic Reed Sternberg (RS) cells and non-neoplastic lymph node components.

Introduction

Secondary malignancies are major treatment-related complications in HL and a leading cause of death of long-term survivors. They may arise from clonal selection of cells that have accumulated transforming genomic lesions induced by chemo- and radiotherapy. DNA repair defects are assumed to increase the susceptibility to treatment-related cancers (1). Hodgkin and Reed-Sternberg (H/RS) cells, the pathognomonic cells of HL, are aberrant post-germinal center B cells that have lost their identity as a consequence of the abolition of B cell gene transcription program and evade apoptotic cell death through CD30 signaling, NF-κB activation and Fas pathway failure (2). Virtually all H/RS cells exhibit neither clonal nor disease-specific chromosomal abnormalities that may originate from their own or their progenitor inherent genomic instability (3). The hypothesis of an unstable genetic background concurrently promoting the evasion of H/RS cell progenitors from FAS-mediated negative selection in the germinal centre, accumulation of multiple genetic aberrations driving their progression towards a fully transformed phenotype and deregulated differentiation culminating in endomitosis is advanced by the findings that chromosomal abnormalities (eventually resulting in onc suppressor loss) are not restricted to the malignant cellular context (4-8). The association of polymorphisms in DNA repair and oxidative stress response genes with an increased risk for non-Hodgkin lymphoma supports a role of individual genetic susceptibility in primary B lymphomagenesis (9,10). However, the determining factors of chromosomal lesions in HL are still elusive. Most likely, they do not include defects of nucleotide mismatch repair resulting in microsatellite instability, a putative source of genomic instability in H/RS cells (11).
Genomic instability in therapy-related myelodysplastic syndromes and AMLs seems genetically determined. It might stem from defects of detoxification systems designed to limit oxidative DNA damage (driven by polymorphisms of genes involved in drug metabolism) and/or chromatin assembly factors, resulting in accumulation of double strand breaks (DSB) and high levels of sister chromatid exchange (SCE) (12,13). More recently, combined polymorphisms in human homeobox HLX1 (a determinant of hematopoietic stem cell frequency) and DNA repair RAD1 genes have been associated with an increased risk of therapy-related AML (14).

We used an aCGH strategy to detect and map genomic imbalances (amplifications and deletions) in whole formalin-fixed, paraffin-embedded lymph nodes of 3 patients with classic HL at diagnosis who developed secondary AML during the follow-up. Our results underscored 3 amplifications and 5 deletions common to all 3 patients. Further investigation is required to elucidate whether those genomic imbalances may be considered as molecular risk factors for secondary leukemogenesis.

Materials and methods
ACGH analysis. DNA extraction was performed using a commercial kit (QIAamp DNA kit from Qiagen) according to manufacturer’s instructions and quantified by means of NanoDrop Technology (Wilmington). Low DNA amounts were amplified by means of DOP-PCR (Roche). GenoSensor array 300 microarrays (Abbott Vysis Inc.) consisted of 287 probes spotted in triplicate. Briefly, test and reference DNAs, arrayed 300 microarrays (Abbott Vysis Inc.) consisted of 287 probes spotted in triplicate. Briefly, test and reference DNAs, after random priming labelling with Cy3-dCTP and Cy5-dCTP (Amersham Biosciences) and electrophoresis resolution in 1.5% agarose gel to confirm their length range between 100 and 600 bp, were denatured at 80°C for 10 min in 25 μl hybridization buffer containing human Cot-I DNA and hybridized on microchips at 37°C for 72 h. After washing microarrays were counterstained with DAPI IV (Abbott Vysis Inc.). The images were captured and analyzed with the GenoSensor reader system (Abbott Vysis Inc.). Only those probes with at least 2 available spots were considered for analysis (spot measurements were highly reproducible, thus two spots are considered sufficient) and the average over the spots was taken as the copy number ratio for that gene. Thresholds for gene amplification and deletion were 1.2 and 0.82, respectively (15).

Fluorescence in situ hybridization (FISH). FISH was performed on sections from formalin-fixed, paraffin-embedded biopsy blocks using the following commercially available fluorescence-labelled, locus-specific (LSI) N-myc and CDKN 2A (p16) and associated centromere DNA probes (CEP) (Abbott Vysis Inc.). Fifty nuclei per sample were scored for each LSI probe. Digital images were acquired with a Nikon Eclipse 1000 fluorescence microscope. Amplifications and deletions were detected when the ratio between the number of spots of test gene and of internal reference gene was greater than 2N+1 or lower than 1 respectively, with N corresponding to the number of spots of the internal reference gene.

Immunohistochemistry. Tissue sections (3 μm) formalin-fixed, paraffin-embedded blocks were coated on electrically charged slides, dewaxed and rehydrated, and then submitted to antigen retrieval by micro-waving in 1 mM EDTA (pH 8.0) at 900 W (3 cycles of 5 min each). After cooling, slides were incubated for 30 min at room temperature with primary antibodies [PPARBP and GATA3 from Santa Cruz Biotech., Top1, FGFR1 and FGR(SRC2) from Abcam, FES from Abgent, HRAS and WT1 from Dako]. Antibody binding was detected by the alkaline phosphatase anti-alkaline phosphatase technique or by the peroxidase-based LSAB method (Dako) and sections were then counterstained with hematoxylin.

Polymerase chain reaction (PCR). PCR reactions were performed on T Personal instrument by Biometra according to the following cycle profile: denaturation at 98°C for 30 sec, annealing at 59°C for 30 sec and extension 72°C for 1 min. Thirty cycles were carried out for PCR amplification of β-actin. Amplification products (226 bp) were then resolved in 1% agar and signal intensities measured by densitometric analysis. β-actin primers sequences are: 5’CATGTGCAAG GCCGGCTTC 3’ (upper) and 3’GAAGGTGTGTGGTGTC CAGATT 5’ (lower). PCR reactions were carried out on a total amount of 500 ng of DOP-PCR (Roche kit) products in PCR reaction buffer containing 30 nM Tris pH 8.4, 250 μg/ml BSA, 3 mM MgCl2, 2 mM dNTPs, 4 U Taq polymerase and 0.5 μM specific primers.

Results and Discussion
The cumulative incidence of secondary acute myeloid leukemias (AML) in HL ranges from 0.8% to 6.3%. It is mostly restricted to the first 5-7 years after treatment and correlated with the genotoxic damage induced by chemotherapeutic protocols including alkylating drugs (mechlorethamine, in particular) (12). In our cohort of 1,224 classic HL patients (corresponding to 0.89%) 11 underwent this therapy-related outcome (data not shown). To investigate whether individual genetic imbalances at diagnosis would distinguish patients prone to AML development during HL follow-up we used an aCGH strategy (GenoSensor array 300 purchased from Abbott Vysis Inc.). In preliminary experiments (data not shown) we did not detect differences in DNA sequence copy numbers of whole lymph node and peripheral blood mono-nuclear cells from 2 normal persons and 2 classic HL patients at diagnosis (with <2% neoplastic component), supporting that aCGH mirror the individual genotypic profiles. However, the putative impact of neoplastic lymph node microenvironment and/or infection (in particular by Epstein-Barr virus) on genomic profiles of diverse lymph node cellular components possibly relevant for HL prognosis requires further investigation in a larger number of patients and using different techniques (aCGH technique only detects amplifications and deletions but does not provide any information on gene expression) (16,17).

Good quality DNA suitable for aCGH analysis was obtained from lymph node biopsies of 3 out of 11 classic HL cases who developed AML (see Table I for clinical details). They were used as tests in a first aCGH series set using pooled DNAs from 8 reactive lymph node biopsies as reference. To confirm the good quality of DNA used in aCGH, PCR reactions for β-actin were performed on DOP-PCR products of all patients involved in the study (3 classic HL and 3
Genomic imbalances included 44 amplifications and 28 deletions. Of 44 amplifications, 24 were common to all 3 patients, 17 to 2 patients and the remaining 3 were seen only in 1 patient (Table IIA). Out of 28 deletions, 5 were common to all 3 patients, 12 were shared by 2 patients and the remaining 11 were seen only in 1 patient (Table IIB).

ACGHs results relative to MYCN (N-Myc) amplification and CDKN2A (p16) deletion were validated by FISH. We analysed ratio between number of spots of test genes [MYCN (N-Myc) and CDKN2A (p16)] and of internal reference genes (cep2 and cep9 respectively) (Fig. 2). Notably, most genomic imbalances shared by at least 2 classic HL patients who developed AML were also found in aCGH set using DNAs.

Table I. Clinical details of classic HL patients included in the study.

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<th>Patient 2</th>
<th>Patient 3</th>
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<td></td>
<td></td>
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<tr>
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<td>Unclassified</td>
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<td>sclerosis</td>
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<tr>
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<td>IV B (bone)</td>
<td>III B</td>
<td>II A (bulky mediastinum)</td>
</tr>
<tr>
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<td>8 MOPP cycles+ 8 ABVD cycles</td>
<td>6 VBM cycles+ 3 MOPP cycles</td>
<td>6 ABVD cycles</td>
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<td>Not done</td>
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<td>Nodular</td>
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<td>Clinical stage at diagnosis</td>
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<td>IV B (lung and spleen)</td>
<td>II A (bulky mediastinum)</td>
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<tr>
<td>Radiotherapy</td>
<td>Not done</td>
<td>Sovraclavear and laterocervical lymph nodes, mediastinum</td>
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Patients 1, 2 and 3 developed AML. Patients 4, 5 and 6, who did not develop any secondary malignancy, were comparable to the first group of patients for histological type, clinical stage and follow-up. *According to FAB classification.

Figure 1. Polymerase chain reaction (PCR) for β-actin. PCR reactions for β-actin were performed in DOP-PCR products of all patients involved in the study. Pooled DNAs from normal persons and the six patients showed comparable levels of β-actin gene, confirming the good quality of DNA used in aCGH.

Figure 2. Results of FISH analysis relative to MYCN (N-Myc) and CDKN2A (p16). FISH analysis was performed on paraffin-embedded lymph node biopsies of normal persons (the only one shown here is representative of all), 3 HL patients who developed AML (bars 1, 2 and 3) and 3 HL patients (comparable for histological type, clinical stage and follow-up) who did not develop any secondary malignancy (bars 4, 5 and 6). FISH was performed on sections from formalin-fixed, paraffin-embedded biopsies as described in Materials and methods. Cep2 and cep9 centromeric regions were used as references for the signal copy number in single nuclei.

Patients 1, 2 and 3 developed AML. Patients 4, 5 and 6, who did not develop any secondary malignancy, were comparable to the first group of patients for histological type, clinical stage and follow-up. *According to FAB classification.
Table II. Genomic imbalances in classic HL patients who developed secondary AML relative to normal controls.

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<th>Genes and regions</th>
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ACGHs were performed using DNA from whole formalin-fixed, paraffin-embedded lymph node biopsies of individual classic HL patients who developed secondary AML at diagnosis as test and pooled DNAs from 8 reactive lymph nodes as reference. Panels A and B show in sequence amplifications and deletions seen in 3, 2 or only 1 HL patient. Bold characters distinguish genes and regions shared by at least 2 out of 3 classic HL patients comparable for histological type, clinical stage and follow-up who did not develop any secondary malignancy (see Table IIIA and B).

Table II. Continued.

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from 3 classic HL patients who did not develop any secondary cancer (comparable for histological type, clinical stage and follow-up) as tests and pooled DNAs from 8 reactive lymph node biopsies as reference (Table IIIA and B). They encompass genes involved in genomic stability and concerning, in particular, telomeric regions (16PTEL03, 20QTEL14, 6qTEL54, 8M16/SP6, INS, EST CDY16, sSGA2796), growth factor and hormone receptors (EGR1, ERBB2, FES, EGR2, ESR1), regulators of cell cycle progression (RB1, CDKN2A), apoptosis (PTGS2 (COX2)) and chromatin epigenetic structure (MLL), and signal transduction signals (HRAS, MYCN, MYB, PIK3CA, MAP2K5). In conclusion, our results suggest a
Table III. Genomic imbalances in HL patients who did not develop any secondary malignancy relative to normal controls.

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Table III. Continued.

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</table>

ACGH were performed using DNA from whole formalin-fixed, paraffin-embedded lymph node biopsies of individual classic HL patients at diagnosis (comparable to those who developed AML for histological type, clinical stage and follow-up) as test and pooled DNAs from 8 reactive lymph nodes as reference. Bold characters distinguish genes and regions shared by at least 2 out of 3 of them.

ACGH results underscored a high degree of variability in genomic imbalances associated with AML development concerning all genes and regions whose copy number relative to normal controls was altered (Table VI). The number of amplifications and deletions in patient 3 (the only one exhibiting complex karyotypic abnormalities in leukemic progenitors) largely exceeded that of wide individual variability in the DNA sequence copy number associated with classic HL, likely independent from further evolution of the disease.

To distinguish genomic imbalances eventually associated with the development of secondary AML we set aCGH using individual DNAs from lymph node biopsies of classic HL patients who developed AML as tests and pooled DNAs from 8 reactive lymph nodes as reference. Bold characters distinguish genes and regions shared by at least 2 out of 3 of them.
Table VI. Genomic imbalances in classic HL patients who developed secondary AML relative to HL patients who did not develop any secondary malignancy.

### A. Amplifications

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### B. Deletions

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<tr>
<td>D6S311</td>
<td></td>
<td>0.89</td>
<td>0.88</td>
<td>0.78</td>
</tr>
</tbody>
</table>

ACGHs were performed using DNA from formalin-fixed, paraffin-embedded lymph node biopsies of individual HL patients who developed AML as test and pooled DNAs from lymph node biopsies of HL patients who did not develop any secondary malignancy (comparable for clinical feature, histological type and follow-up) as reference. Bold characters distinguish genomic imbalances in secondary AML relative to normal controls was found altered (see Table IIA and B).

Table V. Genomic imbalances shared by all 3 HL patients who developed secondary AML relative to HL patients who did not develop any secondary malignancy.

### A. Amplifications

<table>
<thead>
<tr>
<th>Genes and regions</th>
<th>Chromosomal locus</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>APM137XA11</td>
<td></td>
<td>9p11.2</td>
<td>1.67</td>
<td>1.24</td>
</tr>
<tr>
<td>FGFR1</td>
<td></td>
<td>8p11.2-p11.1</td>
<td>1.22</td>
<td>1.29</td>
</tr>
<tr>
<td>PPARBP</td>
<td></td>
<td>17q12</td>
<td>1.37</td>
<td>1.38</td>
</tr>
</tbody>
</table>

### B. Deletions

<table>
<thead>
<tr>
<th>Genes and regions</th>
<th>Chromosomal locus</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>APM217YD10</td>
<td></td>
<td>17q tel</td>
<td>0.65</td>
<td>0.72</td>
</tr>
<tr>
<td>FGFR(SRC2)</td>
<td></td>
<td>1p36.2-p36.1</td>
<td>0.56</td>
<td>0.81</td>
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<tr>
<td>GATA3</td>
<td></td>
<td>10p15</td>
<td>0.75</td>
<td>0.79</td>
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<tr>
<td>TOP1</td>
<td></td>
<td>20q12-q13.1</td>
<td>0.64</td>
<td>0.65</td>
</tr>
<tr>
<td>WT1</td>
<td></td>
<td>11p13</td>
<td>0.59</td>
<td>0.69</td>
</tr>
</tbody>
</table>

See legends to Table IV for details on aCGH.

In conclusion, the number of patients included in our study is too limited to assert that genomic imbalances that we found earn consideration as molecular risk factors for secondary AML in HL. Our results must be confirmed in a larger cohort of HL patients. Moreover, the role of individual genomic imbalances in secondary leukemogenesis process requires further investigation. Previous studies proved that
WT1 and FGR(SRC2) participate in the immune response against leukemic clones and that TOP1 and FGFR1 intervene in DNA repair (18,19). In particular, TOP1 deletion impairs p53-dependent recombination repair by precluding DNA cleavage in the vicinity of lesions and FGFR1 overexpression targets the centrosome where it activates signalling pathways via tyrosine phosphorylation and allows continuous cell cycle progression (20,21). They might, therefore, concurrently promote the selection of genetic aberrations in an early myeloid compartment leading to the emergence of leukemic progenitors whose recognition and lysis by CD4+ T and NKT cells is precluded by WT1 and FGR(SRC2) loss.

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References


