# 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 TRß1; 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.

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## 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 oncosuppressor 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 manifacturer'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, 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 $\mu$l 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 $\mu$m) 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 in 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 ß-actin. Amplification products (226 bp) were then resolved in 1% agar and signal intensities measured by densitometric analysis. ß-actin primers sequences are: 5'CATGTGCAAG GCCGGCTTC 3' (upper) and 3'GAAGGTGTGTGGTGC CAGATTT 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 50 nM Tris pH 8.4, 250 $\mu$g/ml BSA, 3 mM $MgCl_2$, 2 mM dNTPs, 4 U Taq polymerase and 0.5 $\mu$M 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 chemotherapy 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 discrete genomic 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 ß-actin were performed on DOP-PCR products of all patients involved in the study (3 classic HL and 3

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

A, Patients who develop AML

| | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| HL | | | |
| Histological type | Nodular sclerosis | Unclassified | Nodular sclerosis |
| Clinical stage at diagnosis | IV B (bone) | III B | II A (bulky mediastinum) |
| Chemotherapy | 8 MOPP cycles+ 8 ABVD cycles | 6 VBM cycles+ 3 MOPP cycles | 6 ABVD cycles |
| Radiotherapy | Not done | Lomboaortic lymph nodes, spleen | Not done |
| Interval between HL diagnosis and AML outcome | 58 months | 76 months | 25 months |
| AML | | | |
| Morphologic subtype[a] | M1 | M0-M1 | M3 |
| Cytogenetics | Normal karyotype | Normal karyotype | tt(15;17)(q22;q12) del(7)(q22;q32) del(9)(q13;q31) |

B, Patients who did not develop AML

| | Patient 4 | Patient 5 | Patient 6 |
|---|---|---|---|
| HL | | | |
| Histological type | Nodular sclerosis | Nodular sclerosis | Nodular sclerosis |
| Clinical stage at diagnosis | III A | IV B (lung and spleen) | II A (bulky mediastinum) |
| Chemotherapy | 8 MOPP cycles+ 8 ABVD cycles | 6 ABVD cycles+ | 4 ABVD cycles |
| Radiotherapy | Not done | Sovraclavear and laterocervical lymph nodes, mediastinum | Sovraclavear and laterocervical lymph nodes, mediastinum |

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. [a]According to FAB classification.

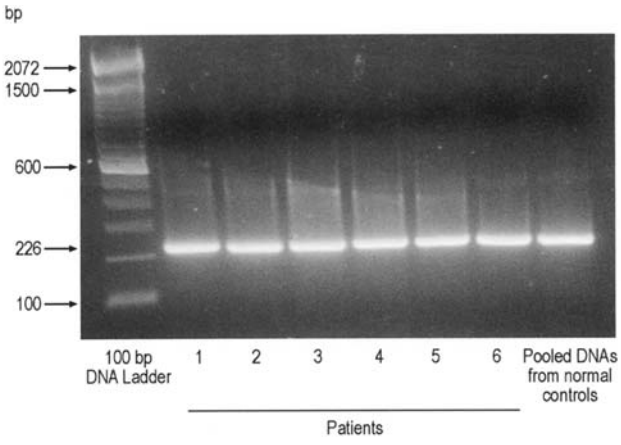


Figure 1. Polymerase chain reaction (PCR) for ß-actin. PCR reactions for ß-actin were performed in DOP-PCR products of all patients involved in the study. Pooled DNAs from normal persons and the six patients showed comparable levels of ß-actin gene, confirming the good quality of DNA used in aCGH.

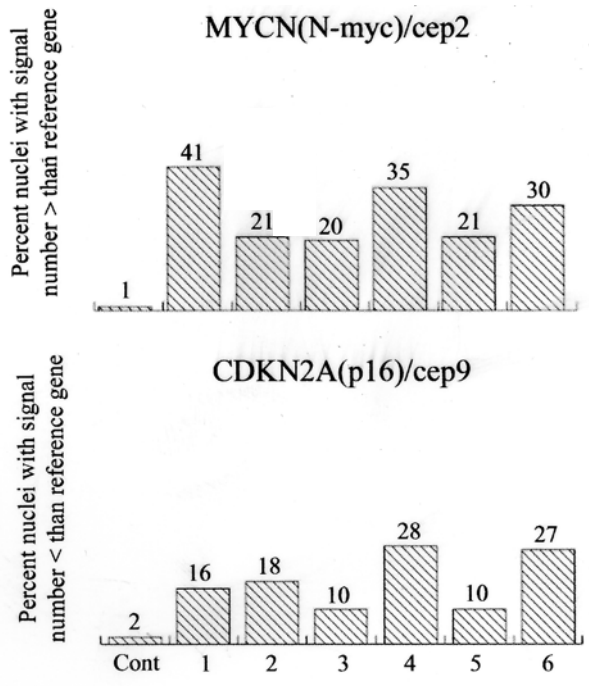


Figure 2. Results of FISH analysis relative to MYCN (N-Myc) and CDKN 2A (p16). FISH analysis was performed on paraffin-embedded lymph node biopsies of normal persons (the only one shown here is representative of all), 3 HL patients who developed AML (bars 1, 2 and 3) and 3 HL patients (comparable for 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.

classic HL who developed AML) and of pooled DNAs from normal persons (Fig. 1). 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 CDKN 2A (p16) deletion were validated by FISH. We analysed ratio between number of spots of test genes [MYCN (N-Myc) and CDKN 2A (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 II. Genomic imbalances in classic HL patients who developed secondary AML relative to normal controls.

A, Amplifications

| Genes and regions | Chromosomal locus | 1 | 2 | 3 |
|---|---|---|---|---|
| 16PTEL03 | 16p tel | 1.22 | 1.42 | 1.66 |
| **20QTEL14** | 20q tel | 1.68 | 1.39 | 2.08 |
| **6QTEL54** | 6q tel | 1.80 | 1.32 | 2.64 |
| 7QTEL20 | 7q tel | 1.29 | 1.43 | 2.04 |
| **8M16/SP6** | 12p tel | 1.50 | 1.46 | 2.68 |
| ARHGAP8 | 22q13.3 | 1.33 | 1.26 | 1.33 |
| ATM | 11q22.3 | 1.24 | 1.26 | 2.12 |
| CDC2L1(p58) | 1p36 | 1.21 | 1.41 | 2.18 |
| **D10S167** | 10p11-10q11 | 1.26 | 1.26 | 2.03 |
| **D13S25** | 13q14.3 | 1.24 | 1.21 | 1.71 |
| **D17S1670** | 17q23 | 1.24 | 1.30 | 1.59 |
| **EGR1** | 5q31.1 | 1.57 | 1.46 | 1.99 |
| **ELN** | 7q11.23 | 1.47 | 1.59 | 2.46 |
| **ERBB2 (HER-2)** | 17q11.2-17q12 | 1.59 | 1.28 | 1.71 |
| **FES** | 15q26.1 | 1.30 | 1.47 | 1.83 |
| **GLI** | 12q13.2-q13.3 | 1.32 | 1.27 | 1.21 |
| **GSCL** | 22q11.21 | 1.53 | 1.23 | 2.04 |
| **HRAS** | 11p15.5 | 2.57 | 1.70 | 3.09 |
| **INS** | 11p tel | 1.30 | 3.00 | 2.68 |
| **MYCN (N-myc)** | 2p24.1 | 1.21 | 1.34 | 1.41 |
| **PTGS2 (COX2)** | 1q31.1 | 1.58 | 1.28 | 2.33 |
| **RB1** | 13q14 | 1.39 | 1.30 | 1.61 |
| **SNRPN** | 15q12 | 1.21 | 1.53 | 2.01 |
| **SRY** | Yp11.3 | 1.35 | 1.29 | 1.86 |
| **D21S378** | 21q11.2 | 1.35 | 1.18 | 1.26 |
| **D5S23** | 5p15.2 | 1.30 | 1.32 | 2.05 |
| **D6S434** | 6q16.3 | 1.33 | 1.05 | 1.84 |
| **D2S447** | 2q tel | 1.88 | 2.54 | 1.06 |
| ABL1 | 9q34.1 | 1.33 | 1.02 | 1.42 |
| **D3S1274, ROBO1** | 3p12-3p13 | 1.33 | 0.90 | 1.61 |
| **DCC** | 18q21.3 | 1.83 | 1.10 | 2.84 |
| BRCA2 | 13q12-q13 | 1.23 | 0.95 | 1.24 |
| **CDK6** | 7q21-22 | 1.30 | 0.71 | 1.54 |
| **E2F5** | 8p22-q21.3 | 2.09 | 1.17 | 2.47 |
| **PIM1** | 6p21.2 | 1.62 | 1.09 | 1.85 |
| **CSF1R** | 5q33-35 | 1.08 | 1.31 | 1.25 |
| **SGC34236** | 2q13 | 1.11 | 1.44 | 2.09 |
| **WHSC1** | 4p16.3 | 1.10 | 1.23 | 1.54 |
| **PDGFB (SIS)** | 22q13.1 | 0.56 | 1.63 | 2.02 |
| OCRL1 | Xq25 | 0.85 | 1.24 | 1.30 |
| **KAL** | Xp22.3 | 1.19 | 1.21 | 1.96 |
| **IGH (SHGC-36156)** | 14q tel | 1.14 | 0.82 | 1.35 |
| **D6S268** | 6q16.3-q21 | 1.18 | 1.00 | 1.47 |
| **D13S319** | 13q14.2 | 1.08 | 1.10 | 2.09 |

Table II. Continued.

B, Deletions

| Genes and regions | Chromosomal locus | 1 | 2 | 3 |
|---|---|---|---|---|
| **EGR2** | 10q21.3 | 0.80 | 0.62 | 0.68 |
| **ESR1** | 6q25.1 | 0.73 | 0.81 | 0.66 |
| **EST CDY16** | X/Yq tel | 0.77 | 0.77 | 0.62 |
| **MLL** | 11q23 | 0.82 | 0.80 | 0.81 |
| **PIK3CA** | 3q26.3 | 0.81 | 0.69 | 0.67 |
| **MAP2K5** | 15q23 | 0.67 | 0.93 | 0.66 |
| RAF1 | 3p25 | 0.81 | 1.02 | 0.75 |
| **MYB** | 6q22-q23 | 0.83 | 0.58 | 0.68 |
| **ABCB1 (MDR1)** | 7q21.1 | 1.00 | 0.55 | 0.72 |
| **CDKN 2A (p16)** | 9p21 | 0.85 | 0.76 | 0.66 |
| **CYP 24** | 20q13.2 | 0.91 | 0.66 | 0.73 |
| **D1S2465** | 1p12 | 0.92 | 0.65 | 0.61 |
| **ITGA4** | 2q31-q32 | 0.91 | 0.62 | 0.76 |
| **SHGC-182** | 1q tel | 1.17 | 0.57 | 0.69 |
| **TGFB2** | 1q41 | 0.90 | 0.79 | 0.73 |
| stSG42796 | 19p tel | 1.13 | 0.57 | 0.73 |
| **TERC** | 3q26 | 1.05 | 0.65 | 0.75 |
| RASSF1 | 3p21.3 | 0.78 | 1.49 | 0.96 |
| **IGH** | 14q tel-2 | 1.14 | 0.82 | 1.35 |
| DAB2 | 5p13 | 0.98 | 0.67 | 0.91 |
| **KAI1** | 11p11.2 | 0.94 | 0.83 | 0.67 |
| **BCL2 3'** | 18q21.3 | 0.92 | 0.83 | 0.65 |
| **D19S238E** | 19q tel | 1.00 | 0.93 | 0.77 |
| **LPL** | 8p22 | 0.94 | 0.89 | 0.65 |
| TCL1A | 14q32.1 | 0.88 | 0.99 | 0.81 |
| ABCC1 (MRP1) | 16p13.1 | 0.88 | 0.95 | 0.72 |
| D6S311 | 6p23-24 | 0.94 | 0.87 | 0.81 |
| TK1 | 17q23.2-q25.3 | 0.98 | 0.90 | 0.75 |

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).

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, stSG42796), 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, MAPK2K5). In conclusion, our results suggest a

Table III. Genomic imbalances in HL patients who did not develop any secondary malignancy relative to normal controls.

A, Amplifications

| Genes and regions | 4 | 5 | 6 |
|---|---|---|---|
| 16PTEL03 | 1.14 | 1.35 | 1.01 |
| **20QTEL14** | 1.12 | 3.08 | 1.87 |
| **6QTEL54** | 1.09 | 3.55 | 1.87 |
| 7QTEL20 | 1.04 | 1.70 | 1.03 |
| **8M16/SP6** | 1.11 | 3.46 | 1.97 |
| ARHGAP8 | 0.95 | 1.21 | 0.96 |
| ATM | 1.16 | 2.81 | 1.06 |
| CDC2L1 (p58) | 1.11 | 1.62 | 1.15 |
| **D10S167** | 1.09 | 1.73 | 1.24 |
| **D13S25** | 1.45 | 1.61 | 2.77 |
| **D17S1670** | 0.92 | 2.20 | 1.36 |
| **EGR1** | 1.69 | 2.64 | 1.23 |
| **ELN** | 1.49 | 2.51 | 1.26 |
| **ERBB2 (HER-2)** | 1.08 | 2.29 | 1.54 |
| **FES** | 1.64 | 1.78 | 2.32 |
| **GLI** | 1.13 | 1.40 | 1.24 |
| **GSCL** | 1.08 | 2.56 | 1.45 |
| **HRAS** | 1.35 | 4.43 | 2.75 |
| **INS** | 1.34 | 3.87 | 2.35 |
| **MYCN (N-myc)** | 1.29 | 1.50 | 1.75 |
| **PTGS2 (COX2)** | 1.03 | 2.61 | 1.82 |
| **RB1** | 1.00 | 2.15 | 1.61 |
| **SNRPN** | 1.20 | 2.20 | 1.43 |
| **SRY** | 1.07 | 1.95 | 1.33 |
| **D21S378** | 1.41 | 1.73 | 2.59 |
| **D5S23** | 1.07 | 3.14 | 1.40 |
| **D6S434** | 1.29 | 2.22 | 2.23 |
| **D2S447** | 4.08 | 0.96 | 2.74 |
| ABL1 | 0.98 | 1.75 | 1.03 |
| **D3S1274, ROBO1** | 1.01 | 2.54 | 1.31 |
| **DCC** | - | 4.45 | 1.63 |
| BRCA2 | 0.84 | 1.51 | 0.92 |
| **CDK6** | 0.92 | 1.86 | 1.29 |
| **E2F5** | 1.09 | 4.08 | 2.29 |
| **PIM1** | 1.17 | 2.95 | 1.66 |
| **CSF1R** | 1.38 | 1.54 | 1.77 |
| **SGC34236** | 1.21 | 2.43 | 1.31 |
| **WHSC1** | 1.25 | 1.53 | 2.17 |
| **PDGFB (SIS)** | 1.24 | 1.56 | 1.13 |
| OCRL1 | 1.16 | 0.95 | 1.36 |
| **KAL** | 0.89 | 2.46 | 1.50 |
| **IGH (SHGC-36156)** | 0.84 | 1.93 | 1.23 |
| **D6S268** | 1.10 | 1.79 | 1.28 |
| **D13S319** | 0.90 | 2.42 | 1.28 |
| AFM217YD10 | 1.36 | 1.66 | 1.88 |
| FGR(SRC2) | 1.44 | 1.29 | 2.20 |
| GATA3 | 1.09 | 1.88 | 1.36 |
| TOP1 | 1.61 | 1.98 | 2.51 |
| WT1 | 1.80 | 1.91 | 2.52 |
| DRIM, ARL1 | 1.21 | 1.28 | 1.31 |
| TBR1 | 1.35 | 1.24 | 1.58 |

Table III. Continued.

B, Deletions

| Genes and regions | 4 | 5 | 6 |
|---|---|---|---|
| **EGR2** | 0.78 | 0.66 | 0.57 |
| **ESR1** | 0.78 | 0.72 | 0.64 |
| **EST CDY16** | 0.69 | 0.70 | 0.53 |
| **MLL** | 0.79 | 0.78 | 0.41 |
| **PIK3CA** | 0.66 | 0.94 | 0.38 |
| **MAP2K5** | 0.76 | 0.68 | 0.55 |
| RAF1 | 1.07 | 0.78 | 0.86 |
| **MYB** | 0.84 | 0.67 | 0.53 |
| **ABCB1 (MDR1)** | 0.82 | 0.63 | 0.46 |
| **CDKN 2A (p16)** | 0.71 | 0.67 | 0.55 |
| **CYP 24** | 0.80 | 0.66 | 0.46 |
| **D1S2465** | 0.72 | 0.61 | 0.53 |
| **ITGA4** | 0.81 | 0.74 | 0.73 |
| **SHGC-182** | 0.68 | 0.60 | 0.42 |
| **TGFB2** | 0.81 | 0.75 | 0.63 |
| stSG42796 | 0.83 | 0.90 | 0.76 |
| **TERC** | 0.99 | 0.72 | 0.65 |
| RASSF1 | 1.21 | 0.50 | 0.94 |
| **IGH** | 0.86 | 0.63 | 0.56 |
| DAB2 | 0.87 | 0.85 | 0.62 |
| **KAI1** | 0.90 | 0.79 | 0.49 |
| **BCL2 3'** | 0.79 | 0.62 | 0.47 |
| **D19S238E** | 1.01 | 0.69 | 0.79 |
| **LPL** | 0.77 | 0.68 | 0.64 |
| TCL1A | 0.97 | 0.70 | 0.83 |
| ABCC1 (MRP1) | 1.12 | 0.70 | 0.96 |
| D6S311 | 1.08 | 0.78 | 1.02 |
| TK1 | 0.91 | 0.85 | 0.59 |

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.

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 lymph nodes of 3 HL patients (comparable for histological type, clinical stage and follow-up) who did not develop any secondary malignancy as reference. 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

Table VI. Genomic imbalances in classic HL patients who developed AML relative to HL patients who did not develop any secondary malignancy.

A, Amplifications

| Genes and regions | 1 | 2 | 3 |
|---|---|---|---|
| 6QTEL54 | 0.81 | 0.88 | **1.41** |
| 7QTEL20 | 0.91 | **1.23** | **1.38** |
| 8M16/SP6 | 0.79 | 0.89 | **1.28** |
| ATM | 0.96 | 0.93 | **1.59** |
| CDC2L1 (p58) | 0.75 | 0.91 | **1.31** |
| D10S167 | 0.87 | 1.02 | **1.34** |
| D17S1670 | 0.81 | 0.99 | **1.21** |
| EGR1 | 0.80 | 0.87 | **1.25** |
| ELN | 0.74 | 0.97 | **1.39** |
| ERBB2 (HER-2) | 0.86 | 1.07 | **1.35** |
| GSCL | 1.01 | 1.04 | **1.44** |
| HRAS | **4.73** | 1.01 | 1.08 |
| INS | 0.85 | **1.28** | **1.35** |
| PTGS2 (COX2) | 1.05 | 0.96 | **1.72** |
| RB1 | 0.93 | 0.95 | **1.47** |
| SNRPN | 0.92 | 0.93 | **1.78** |
| SRY | 0.79 | 0.83 | **1.22** |
| D21S378 | 0.87 | 0.68 | **1.21** |
| D5S23 | 1.08 | 0.99 | **1.64** |
| D3S1274, ROBO1 | 1.00 | 0.87 | **1.53** |
| CDK6 | 1.01 | 0.99 | **1.51** |
| E2F5 | 0.79 | 0.92 | **1.32** |
| PIM1 | 0.89 | 0.94 | **1.36** |
| SGC34236 | 0.81 | 0.96 | **1.37** |
| IGH (SHGC-36156) | 1.04 | 0.92 | **1.21** |
| D6S268 | 0.88 | 0.89 | **1.24** |
| D13S319 | 0.86 | 0.99 | **1.57** |

B, Deletions

| Genes and regions | 1 | 2 | 3 |
|---|---|---|---|
| EGR2 | 1.28 | **0.79** | **0.61** |
| ESR1 | 1.08 | **0.76** | **0.58** |
| EST CDY16 | 1.31 | **0.81** | **0.69** |
| MAP2K5 | 1.18 | 0.96 | **0.60** |
| CDKN 2A (p16) | 1.26 | **0.80** | 0.84 |
| CYP 24 | 1.32 | 0.94 | **0.81** |
| D1S2465 | 1.14 | 0.86 | **0.50** |
| TGFB2 | 1.05 | 0.96 | **0.70** |
| BCL2 3' | 1.11 | 1.12 | **0.81** |
| LPL | 1.03 | 1.06 | **0.81** |
| ABCC1 (MRP1) | 0.99 | 1.18 | **0.73** |
| D6S311 | 0.89 | 0.88 | **0.78** |

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 individual patients. In all cases those genomic imbalances concern genes and regions whose copy number relative to normal controls was found altered (see Table IIA and B).

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

A, Amplifications

| Genes and regions | Chromosomal locus | 1 | 2 | 3 |
|---|---|---|---|---|
| AFM137XA11 | 9p11.2 | 1.67 | 1.24 | 1.59 |
| FGFR1 | 8p11.2-p11.1 | 1.22 | 1.29 | 1.30 |
| PPARBP | 17q12 | 1.37 | 1.38 | 1.21 |

B, Deletions

| Genes and regions | Chromosomal locus | 1 | 2 | 3 |
|---|---|---|---|---|
| AFM217YD10 | 17q tel | 0.65 | 0.72 | 0.66 |
| FGR(SRC2) | 1p36.2-p36.1 | 0.56 | 0.81 | 0.66 |
| GATA3 | 10p15 | 0.75 | 0.79 | 0.61 |
| TOP1 | 20q12-q13.1 | 0.64 | 0.65 | 0.72 |
| WT1 | 11p13 | 0.59 | 0.69 | 0.68 |

See legends to Table IV for details on aCGH.

seen in patients 1 and 2 (Table I). Genomic imbalances shared by all 3 patients included 3 amplifications, encompassing genes and regions whose copy numbers relative to normal controls were not altered, and 5 deletions, due to the loss of amplification seen in classic HL patients who did not develop secondary leukemia) (Table VA and B).

To confirm aCGH results and distinguish the distribution of either amplified or deleted gene product in different lymph node components we performed immunohistochemistry analysis. The results of immunohistochemistry analyses in lymph node biopsies of patients #2 and #6 shown in Fig. 3 are representative of all. A significant reduction of the expression of FGR(SRC2) and TOP1 proteins, apparent in lymph node biopsies from HL patients who developed secondary AML compared to those who did not develop any secondary malignant neoplasia, concerned both the neoplastic (RS cells) and non-neoplastic lymph node components (Fig. 3). Conversely, the expression of WT1 protein was lacking in RS cells of either patient group and significantly reduced in non-neoplastic components of lymph node biopsies of HL patients who developed secondary AML. Furthermore, FGFR1 overexpression in lymph node biopsies of HL patients who developed AML concerned the non-neoplastic lymph node components. Results relative to PPARBP amplification and GATA3 deletion were impaired by the inadequate antibody specificity.

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

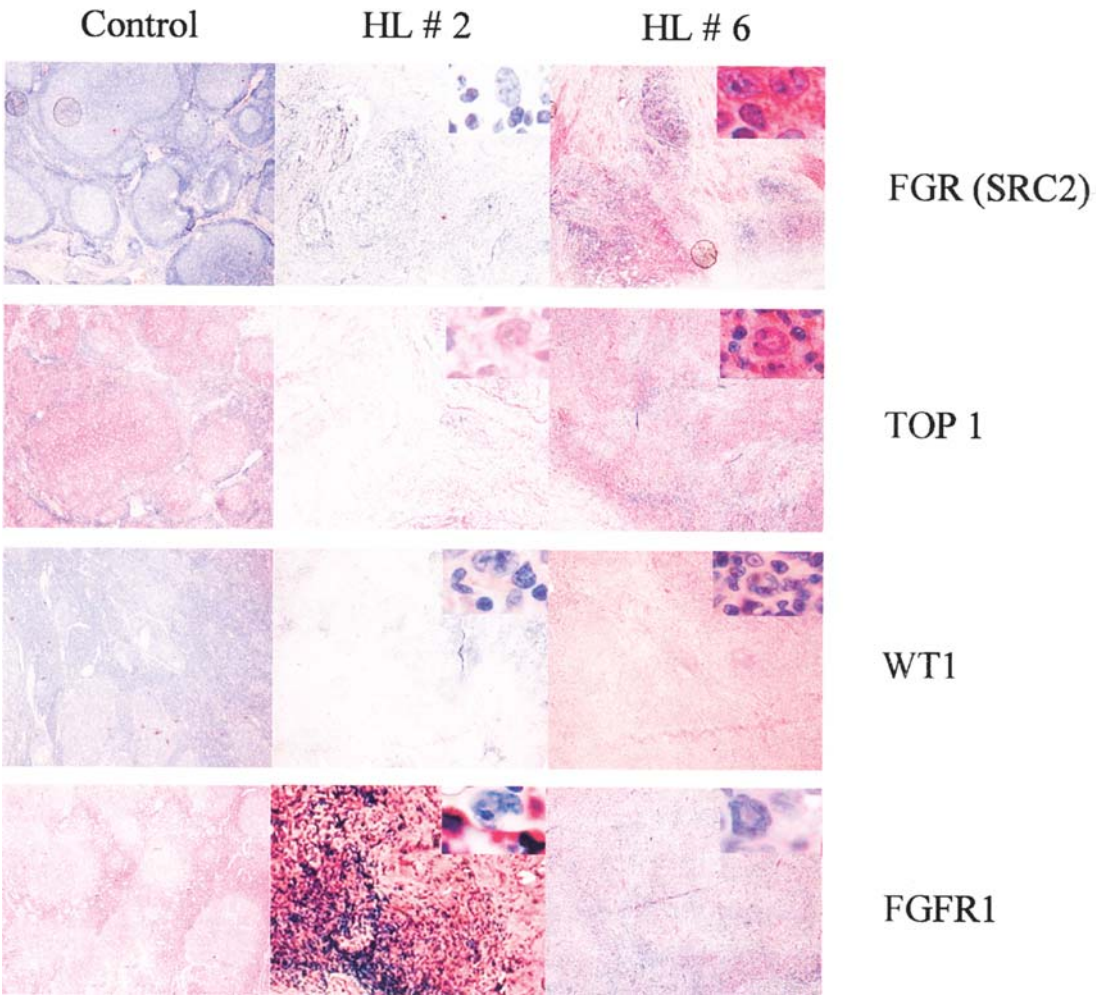


Figure 3. Immunohistochemistry of lymph node biopsies from normal persons and classic HL patients. The panels show in sequence FGR(SRC2), TOP1, WT1 and FGFR1 expression in lymph node biopsies from a normal control (reactive lymph node biopsy), one classic HL patient who developed AML (#2) and one classic HL patient who did not develop any secondary malignancy (#6). The expression of above mentioned proteins in RS cells are represented in detail in the inserts at the top of the single panels. Results were confirmed in lymph node biopsies of other normal controls and HL patients. Immunohistochemistry analyses were performed on formalin-fixed, paraffin-embedded tissue sections according to the method described in detail in Materials and methods.

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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