Molecular Karyotypes of Hodgkin and Reed–Sternberg Cells at Disease Onset Reveal Distinct Copy Number Alterations in Chemosensitive versus Refractory Hodgkin Lymphoma

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Abstract

Purpose: To determine the recurring DNA copy number alterations (CNA) in classical Hodgkin lymphoma (HL) by microarray-based comparative genomic hybridization (aCGH) using laser capture microdissected CD30+ Hodgkin and Reed–Sternberg (HRS) cells.

Experimental Design: Archived tissues from 27 CD30+ HL plus control samples were analyzed by DNA microarrays. The HL molecular karyotypes were compared with the genomic profiles of germinal center B cells and treatment outcome (chemotherapy responsive vs. primary refractory disease).

Results: Gains and losses observed in more than 35% of HL samples were localized to 22 and 12 chromosomal regions, respectively. Frequent gains (>65%) were associated with growth and proliferation, NF-κB activation, cell-cycle control, apoptosis, and immune and lymphoid development. Frequent losses (>40%) observed encompassed tumor suppressor genes (SPRY1, NELL1, and ID4, inhibitor of DNA binding 4), transcriptional repressors (TXNIP, thioredoxin interacting protein), SKP2 (S-phase kinase-associated protein 2; ubiquitin ligase component), and an antagonist of NF-κB activation (PPARGC1A). In comparison to the germinal center profiles, the most frequent imbalances in HL were losses in 5p13 (AMACR, GDNF, and SKP2), and gains in 7q36 (SHH, sonic hedgehog homolog) and 9q34 (ABL1, CDK9, LCN2, and PTGES). Gains (>35%) in the HL chemoresponsive patients housed genes known to regulate T-cell trafficking or NF-κB activation (CCL22, CX3CL1, CCL17, DOK4, and IL10), whereas the refractory samples showed frequent loss of 4q27 (interleukin; IL21/IL2) and 17p12, and gain of 19q13.3 (BCL3/RELB).

Conclusion: We identified nonrandom CNAs in the molecular karyotypes of classical HL. Several recurring genetic lesions correlated with disease outcome. These findings may be useful prognostic markers in the counseling and management of patients and for the development of novel therapeutic approaches in primary refractory HL. Clin Cancer Res; 17(10); 3443–54. © 2011 AACR.

Introduction

The annual incidence of Hodgkin lymphoma (HL) is estimated at 3 cases per 100,000 persons, making this malignancy one of the most common lymphomas in the Western world (1). The characteristic pathologic feature of classical HL is the presence of Hodgkin and Reed–Sternberg (HRS) cells, which usually comprise less than 3% of the affected mixed cellular lesion. There is compelling evidence suggesting that the pathognomonic HRS cells are an outgrowth of a malignant clone derived from a "reprogrammed" germinal center (GC) B cell that no longer expresses B-lineage–specific genes, such as POL2F2, POL2AF1, and PL1, and may express genetic markers characteristic of other hematopoietic lineages like IL21 (interleukin), CCL17, CSF1, ID2, CD3, and CD4 (2–4). Recurrent genetic lesions in critical hematopoietic transcription factors have led to the discovery that constitutive activation of the NF-κB signaling pathway is essential for HRS cell survival and proliferation (4). In particular, gains of REL and deletions or inactivating mutations of TNFAIP3 (TNF-α–induced protein 3), gene involved in the NF-κB signaling pathway, have been detected in about 40% of classical HL (5–7). To gain further insight into the pathogenesis of HL and its lineage infidelity, comprehensive genomic characterization of HRS cells from primary HL tumor samples is necessary; however, the scarcity of HRS cells in the HL lesions remains a key limiting factor in unraveling the molecular consequences of this malignancy.
Slovak et al. investigate genome-wide DNA alterations in lesions of Hodgkin lymphoma (HL) using microarray-based comparative genomic hybridization (aCGH) technology. The molecular data show a clear relationship between copy number alterations (CNA) and the genetic changes in HL, including stem cell transplantation, primary refractory HL (PR HL), and relapsed HL (10–13). Distinguishing primary genetic alterations in HL remains a challenge. Recently, high-resolution cytogenetic analysis has provided new opportunities to understand the biology of HL. We provide a comparison of chemo-sensitive and primary refractory HL samples and describe potentially pathogenetic and prognostic CNA differences at disease onset. In particular, HL samples that showed losses of 11p14.3 and gains of 1q24-q31 were associated with primary refractory HL, whereas HL samples showing 16q13 gains or few genetic aberrations were most frequently associated with favorable IPS (international prognostic scores) and chemosensitive phenotypes. Due to the limited number of well-characterized HL samples available for ongoing studies, especially for primary refractory HL, collaboration among lymphoma clinical investigators is imperative to refine, and build on these promising clinical predictors.

**Translational Relevance**
We show the potential of identifying copy number alterations (CNA) in HL by aCGH using DNA extracted from microdissected CD30+ Hodgkin and Reed–Sternberg (HRS) cells. The molecular data show a clear relationship between CNAs of genes associated with the NF-kB signaling pathway, the tumor microenvironment, cell-cycle regulation, and apoptosis. We provide a comparison of chemo-sensitive and primary refractory Hodgkin lymphoma (HL) samples and describe potentially pathogenetic and prognostic CNA differences at disease onset. In particular, HL samples that showed losses of 11p14.3/SLC17A6 and 17p12, with gains of BCL3/19q13.31 were associated with primary refractory HL, whereas HL samples showing 16q13 gains or few genetic aberrations were most frequently associated with favorable IPS (international prognostic scores) and chemosensitive phenotypes.

**Materials and Methods**

**Patient and control samples**

On approval from the Institutional Review Board of the City of Hope, primary formalin-fixed paraffin-embedded (FFPE) diagnostic samples were obtained from 27 patients including 15 patients with CR HL and 12 patients with PR HL. Clinicopathologic characteristics of the patients are summarized in Table 1. The international prognostic scores (IPS) of our study population were calculated as described (16). In this study, patients with an IPS of 2 or less were given a favorable designation and patients with an IPS of 3 or above were assigned to the unfavorable group. Control samples included 9 FFPE benign lymph node samples (4 males and 5 females) and GC cells from 10 FFPE reactive follicular hyperplasia (RFH) samples (2 males and 8 females). Each sample was submitted for conventional histopathologic processing to confirm HL involvement in the test samples or no evidence of malignancy in the control samples.

**FFPE tissue processing and laser capture microdissection**

Five-micrometer serial sections from the FFPE tissue blocks were fixed onto PALM membrane slides (PEN-membrane; Zeiss) and processed as previously described (13, 14). A series of experiments designed to assess the impact of DNA source (e.g., archival material including frozen and FFPE), quantity, and amplification on array CGH was carried out to establish the FFPE aCGH protocol (13). Briefly, the slides were pretreated as follows for immunostaining: 1 hour at 65°C in a dry oven, 1 minute in xylene at room temperature, 5 minutes in 100% ethanol (2×), 5 minutes in 3% H2O2, and rinsed in dH2O. Antigen retrieval was carried out at 98°C for 30 minutes using the decloaking chamber and decloaker universal heat retrieval buffer (both from Biocare Medical). Test sample slides were stained with monoclonal mouse anti-human CD30 (Dako Inc.) using the Dako-Cytomation Autostainer (Dako Inc.) per manufacturer’s instructions. Control samples were stained with CD20. A total of 150 CD30+ HRS cells per test sample and 150

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CD20+ GC cells per control sample were LCM into microfuge caps containing water.

**Whole-genome amplification**
Isolated LCM cells were randomly fragmented and amplified in the LCM collection tubes using the GenomePlex Single-Cell Whole-Genome Amplification Kit (WGA4; Sigma-Aldrich) according to product instructions. The quality and quantity of the isolated amplified DNA were assessed electrophoretically and spectrophotometrically. Samples were processed prior to aCGH using the GenElute PCR Clean-Up Kit (Sigma-Aldrich). A single sample was run in triplicate to evaluate assay reproducibility.

**BAC-based aCGH labeling and hybridization**
One microgram of genomic DNA was labeled using Bioarray Kit (Enzo Life Sciences) prior to subsequent hybridization to a RPCI 19K BAC array (Roswell Park) using a Gentac hybridization station (Genomic Solutions). Sex-mismatched pooled DNA from 20 healthy donors was used as reference DNA. The minimal tiling RPCI BAC array containing about 19,000 BAC clones was used (backbone consisted of ~4,600 BAC clones with a BAC center-to-center distance of 165 kb; human genome build 36.1; ref. 13). Slides were scanned on the GenePix 4200A Scanner (Molecular Devices).

**Control assays, selection criteria, and outcome predictor**
Two control studies were conducted to establish experimental reproducibility and quality control associated with multiple features of our study. These control studies had the objective of establishing standards for processing FFPE tissue, WGA, or eliminate problematic microarray calls associated with cross-hybridizing BACs or poor labeling/hybridization efficiency. First, 9 FFPE benign lymph node controls were processed in a manner identical to the test samples. Second, 1.05 ng of peripheral blood DNA (DNA equivalent of 150 cells) obtained from 6 normal, healthy individuals (3 males and 3 females) was amplified and analyzed by aCGH. Deviant calls from either of these control studies found in 2 or more microarrays were masked before the HL test samples were processed. Selection criteria for the reportable CNA calls in the HL samples were more than 5 BACs (>700 kb in size) observed in 35% or more in either the sensitive or resistant samples with a value of \( P < 0.15 \) and not present in less than 20% (no more than 1 patient) of the benign control samples.

To determine which genes are presumably critical in the HL malignant process, GC B cells from 10 lymph node samples showing RFH were processed identically to the test samples and compared with the HL CNAs. Genes associated with RFH had to meet the following selection criteria: large (>700 kb) CNAs observed 15% or more commonly in the RFH group (2 or more samples) with a value of \( P < 0.15 \) and observed in less than 20% of the benign control group. The CNAs associated with RFH were filtered prior to the comparative analysis of the chemosensitive and primary refractory disease CNAs. This final analysis generated a list of genes that could be used as a potential outcome predictor to discriminate between CR HL and PR HL at presentation. To test the outcome predictor in a preliminary manner, 4 additional primary HL lesions were analyzed in a blinded yet similar manner to the original 27 HL test samples and categorized using the proposed predictor model.

<table>
<thead>
<tr>
<th>Table 1. Clinical and pathologic characteristics of the HL patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Age(^a) (range), y</td>
</tr>
<tr>
<td>Gender (M/F)</td>
</tr>
<tr>
<td>REAL/WHO classification</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
</tr>
<tr>
<td>Mixed cellularity</td>
</tr>
<tr>
<td>Classical Hodgkin’s disease, NOS</td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>Early (I–II)</td>
</tr>
<tr>
<td>Advanced (IIA–IVB)</td>
</tr>
<tr>
<td>A versus B symptoms</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>Bulky disease</td>
</tr>
<tr>
<td>Bone marrow involvement</td>
</tr>
<tr>
<td>IPS score(^b)</td>
</tr>
<tr>
<td>Favorable</td>
</tr>
<tr>
<td>Unfavorable</td>
</tr>
<tr>
<td>Indeterminate</td>
</tr>
<tr>
<td>Initial treatment</td>
</tr>
<tr>
<td>ABVD</td>
</tr>
<tr>
<td>Stanford V</td>
</tr>
<tr>
<td>BEACOPP(^c)</td>
</tr>
<tr>
<td>COPP/ABV</td>
</tr>
<tr>
<td>Treatment response</td>
</tr>
<tr>
<td>Sensitive</td>
</tr>
<tr>
<td>Refractory</td>
</tr>
<tr>
<td>Transplant</td>
</tr>
<tr>
<td>Autologous</td>
</tr>
<tr>
<td>Allogeneic</td>
</tr>
</tbody>
</table>

Abbreviation: NOS, not otherwise specified.
\(^a\)Age, median (y).
\(^b\)IPS is based on 7 potential unfavorable features at diagnosis: serum albumin \(\leq 4\) g/dL (40 g/L), hemoglobin \(\leq 10.5\) g/dL (105 g/L), male gender \(\geq 45\) years, stage IV, WBC \(\geq 15,000\) /\(\mu\)L, and lymphocyte count \(\geq 600\) /\(\mu\)L, and/or \(\geq 8\%\) of the WBC count (15).
\(^c\)Pediatric protocol CCG-59704.

CD20+ GC cells per control sample were LCM into microfuge caps containing water.
Data analysis

Data images were quantified by ImaGene (BioDiscovery, Inc.), and the chromosome regions of abnormal copy number across the genome were detected by Nexus (BioDiscovery, Inc.). To explore gene function and interactions, the CNAs were uploaded into Ingenuity Pathway Analysis tools 3.0 (IPA; http://www.ingenuity.com) for alternative and canonical pathway mapping. IPA calculates a per network significance score by applying the hypergeometric distribution calculated via the Fisher’s exact test for 2 × 2 contingency tables. The P value is calculated by comparing the number of user-specified genes of interest that participate in a given pathway, relative to the total number of occurrences of these genes in all pathway annotations stored in the Ingenuity Pathways Knowledge Base (IPKB).

FISH confirmation studies

Locus-specific FISH analyses were conducted to confirm the recurring CNAs detected by aCGH. The probes used for FISH analysis were carefully chosen and mapped within the genomic coordinates of the specific chromosome region showing gain or loss. In each case, a control DNA FISH probe from the opposite chromosome arm was included to confirm the CNAs in relation to the ploidy level of the HRS cell. To validate CNAs by FISH, CD30-stained IHC slides with AEC detection were scanned, localized, and recorded on the slide using the Bioview Duet Image Analyzer (BioView, Ltd.) prior to FISH analysis as described previously (17).

Statistical evaluation

We background corrected and normalized the quantified images with print-tip loess after removing bad spots. We evaluated all pairwise correlations between normalized replicates by scatter plots and Pearson’s correlations on the log2 ratios.

Results

Defining the threshold and reproducibility of CNAs

From each patient and control FFPE sample, 150 cells (CD30⁺ HRS cells from test samples and CD20⁺ cells from control samples) were isolated from 5-μm thick tissue sections by LCM, amplified using WGA, and hybridized to a 19K whole-genome BAC array. Two control studies were conducted to reduce deviant calls associated with poor hybridization efficiency or amplification bias associated with WGA or FFPE tissues. In the first experiment, 1.05 ng of peripheral blood DNA (DNA equivalent of 150 cells) obtained from 6 normal individuals (3 males and 3 females) were WGA amplified and analyzed by aCGH. In the second study, 9 formalin-fixed benign lymph node control samples (4 males and 5 females) were processed similar to the test samples. A total of 235 of the 16,952 autosomal BACs showed a false-positive (gain or loss) result in the normal control samples; these BACs were masked from the test data analyses. In addition, no chromosomal site was called positive if its respective BACs masked from the test data analyses. In addition, no chromosomal site was called positive if its respective BACs showed gain or loss in more than 2 arrays from normal control samples. On the basis of these control data, and to minimize the risk of reporting false-positive results, thresholds for calling gains and losses were set at more than 35% of test samples. Reproducibility of the FFPE was carried out by analyzing 150 HRS cells from a single HL patient that were isolated independently from the block and processed by the LCM-WGA-aCGH method in triplicate. Supplementary Figure S1 shows the high correlation between the normalized log2 ratio values of each possible pair of replicates (Pearson r = 0.90, P values uniformly <0.0001), confirming reproducibility of the assay and the array.

CNAs detected in 27 primary FFPE diagnostic HL samples

Primary tissue blocks were obtained from 15 CR and 12 PR patients. The clinical and pathologic characteristics of the patients are listed in Table 1. Recurring chromosome imbalances were observed among all 27 HL samples. The most frequent gains (>65%) mapped to 2q37.3, 7p21.1, 8q24.3, 16q23.3-q24.3, 9q34.13-q34.3, 14q32.33, 19q13.33, and 20q13.33 (Supplementary Data; Table 1) and included genes associated with growth and proliferation (AHR, BAI1, BOP1, CNRMD, FOXC2, ITGAV, PTP4A3, SLURP1, CBFA2T3, SLC7A5, NOTCH1, FOXP1, TRAF2, IRF8, S100B, and MYH14), cell cycle (AKT1, CDK10, and SUIMOS), drug metabolism (CYP1B1, CYP1B2, and SLC19A1), angiogenesis and cell adhesion (COL18A1, CDH4, ITGB2, and FOXC2), apoptosis regulation (FOXP1 and GPR132), immune and lymphatic development (CBFA2T3, IL17C, IRF8, CLEC11A, RXRA, SP1B, and ICOSLC) and invasion, metastasis or cancer relatedness (AVAV2, PSCA, PTP4A3, GINS2, FUT7, TUBB2C, KLK, POLD1, and TFF2). Losses observed in more than 40% of HL samples encompassed SPRY1, NELL1, SLC1A3, CENF, IL7R, SKP2 (S-phase kinase-associated protein 2), ID4 (inhibitor of DNA binding 4), PPARGC1A, and TNXIP (thioredoxin interacting protein). Because ploidy differences are inherent to HRS cells, to accurately validate the CNAs observed by aCGH, HRS-specific FISH confirmation studies using locus-specific probes that mapped within the genetic coordinates of the recurring CNAs were paired with a DNA control probe that localized to the opposite chromosome arm. These chromosome region–specific FISH analyses confirmed the presence or absence of aCGH-defined CNAs in CD30⁺ HRS for 15 regions in 3 to 5 patient samples for each paired FISH probe set (Fig. 1).

Comparison of aCGH CNAs in 15 chemosensitive and 12 PR HL samples

The genomic differences observed at more than 35% in the CR HL patients clustered within 2 chromosomal bands on chromosome 16 (16q13 and 16p11.2) and showed gains of genes known to regulate T-cell trafficking or NF-κB activation (CCL22, CX3CL1, CCL17, DOK4, and IL10; Table 2). In comparison to the CR samples, the 12 PR patients showed frequent loss of genes localized to 1q21.2, 4q27, 5p15, 11p14.3, and 17p12 with recurrent gains of chromosome bands 2q37.2, 13q31.2, 19q13.3, and...
22q13.33. These data were also compared with the IPS of our study population (Table 1). Of interest, the 39 unique genes we identified in regions with CNAs comparing patients with favorable versus unfavorable IPS scores showed remarkable overlap with the 63 unique genes identified in chemosensitive and primary resistant genomic profiles (Table 2), with 36 unique genes appearing in both lists. The probability of this occurring by chance follows a hypergeometric distribution (18, 19), and the associated P value was less than 0.0001.

Comparison of HL samples with benign RFH

To delineate which genes are more likely to play a critical role in HL oncogenesis, normal GC cells from 10 RFH samples were analyzed and compared with the HL results. As described in Materials and Methods, strict criteria were established to call recurring gains and losses associated with CR HL, PR HL, and benign RFH. Figure 2A shows the number of genes concordant and discordant among these 3 groups observed in more than 35% of the samples tested in each group. The first assessment of these data identified 151 genes frequently altered in both CR HL and PR HL samples but not detected in the benign RFH samples, 81 genes commonly detected in the CR HL and 312 genes more frequently observed in the PR HL tumors and not in the other 2 groups. The 10 most frequent genetic alterations detected among the 151 HL CNAs included losses of genes in 5p13 (AMACR, GDNF, and SKP2), gains of 7q36 (SHH, sonic hedgehog homolog), and gains of 9q34 (ABL1, CDK9, ENG, LCN2, PTGES, and TSC1). A second assessment of the
In this analysis, 19 genes were clearly more common in chemosensitive samples, whereas 44 genes were more prevalent in the primary refractory tumors (Table 2).

**CNAs interaction networks and predictive modeling**

Possible genetic interactions for those genes listed in Table 2 were examined in the context of the curated list of published molecular interactions in IPKB. In addition to an association with the NF-kB signaling pathway, IPA revealed key genes from these connections between genes in the calcium signaling and BCL3/Ca2+ signaling pathway, IPA revealed predilection for predicting chemosensitive or primary refractory disease.

**Table 2. Consistent CNAs observed in CR HL versus PR HL, not observed in GC cells**

<table>
<thead>
<tr>
<th>Band</th>
<th>Region</th>
<th>Event</th>
<th>Length, bp</th>
<th>Frequency in refractory</th>
<th>Frequency in sensitive</th>
<th>Genes in sensitive points</th>
</tr>
</thead>
<tbody>
<tr>
<td>16q13</td>
<td>chr16:55,456,619-56,127,978</td>
<td>Gain</td>
<td>671,359</td>
<td>25%</td>
<td>60%</td>
<td>ARL2BP, CCDC102A, TCL17, TCL22, CETP, CIAPIN1, COQ9, CPNE2, CX3CL1, DOK4, HERPUD1, NIP30, NLR5C5, PLLP, POLR2C, RSPRY1, SLC12A3</td>
</tr>
<tr>
<td>16p11.2</td>
<td>chr16:32,592,341-33,172,220</td>
<td>Loss</td>
<td>579,879</td>
<td>17%</td>
<td>53%</td>
<td>LOC729355, TP53T3G</td>
</tr>
<tr>
<td>1q21.2</td>
<td>chr1:148,603,613-148,818,760</td>
<td>Loss</td>
<td>215,147</td>
<td>42%</td>
<td>7%</td>
<td>ADAMTSL4, ECM1, KIAA0460, MCL1, TAR5</td>
</tr>
<tr>
<td>2q37</td>
<td>chr2:236,067,474-236,698,859</td>
<td>Gain</td>
<td>631,385</td>
<td>50%</td>
<td>27%</td>
<td>SLC17A6</td>
</tr>
<tr>
<td>4q27</td>
<td>chr4:120,634,997-123,761,662</td>
<td>Loss</td>
<td>3,126,665</td>
<td>50%</td>
<td>20%</td>
<td>AAD1D1, ANXA5, BBS7, C4orf31, CCNA2, EXOS9, GPR103, IL2, IL21, KIAA1109, MAD2L1, PDE5A, TMEM155, TNP3, TRPC3</td>
</tr>
<tr>
<td>5p15.31</td>
<td>chr5:7,449,342-7,883,194</td>
<td>Loss</td>
<td>433,852</td>
<td>42%</td>
<td>0%</td>
<td>ADCY2</td>
</tr>
<tr>
<td>5p15.2</td>
<td>chr5:9,088,137-9,883,463</td>
<td>Loss</td>
<td>595,326</td>
<td>42%</td>
<td>7%</td>
<td>SEMA5A, TAS2R1</td>
</tr>
<tr>
<td>11p14.3</td>
<td>chr11:22,171,297-22,257,975</td>
<td>Loss</td>
<td>86,878</td>
<td>58%</td>
<td>7%</td>
<td>TMEM16E</td>
</tr>
<tr>
<td>13q31.2</td>
<td>chr13:87,122,870-87,129,871</td>
<td>Gain</td>
<td>7,001</td>
<td>58%</td>
<td>27%</td>
<td>SLC17A6</td>
</tr>
<tr>
<td>17p12</td>
<td>chr17:11,864,859-15,526,918</td>
<td>Loss</td>
<td>3,682,059</td>
<td>58%</td>
<td>13%</td>
<td>ADAMTSL4, ECM1, KIAA0460, MCL1, TAR5, CDRT15, CDRT4, COX10, ELAC2, FAM18B2, FLJ45831, HS3ST3A1, HS3ST3B1, MAP2K4, MYOCO, PMP22, RICH2, TEKT3, TRIM16</td>
</tr>
<tr>
<td>19q13.31</td>
<td>chr19:49,894,179-49,955,141</td>
<td>Gain</td>
<td>60,944</td>
<td>50%</td>
<td>20%</td>
<td>BCL3, RELB, CEACAM1</td>
</tr>
<tr>
<td>22q13.33</td>
<td>chr22:49,092,963-49,100,010</td>
<td>Gain</td>
<td>7,047</td>
<td>50%</td>
<td>20%</td>
<td>FAM116B</td>
</tr>
</tbody>
</table>

CR HL and PR HL gene subsets was conducted requiring a frequency difference of more than 20% and a value of \( P = 0.15 \) between the sensitive and resistant samples (Fig. 2B). In this analysis, 19 genes were clearly more common in chemosensitive samples, whereas 44 genes were more prevalent in the primary refractory tumors (Table 2).

On the basis of the relatively high frequency of \( IL21/IL2 \) loss at 4q27, \( BCL3 \) overrepresentation in our PR HL cohort, we hypothesized samples without gains in 16q13 and losses of \( IL21/IL2 \) at 4q27, 11p14.3/SLC17A6, or 17p12 with or without concurrent gains of \( BCL3 \) portend a highly likely drug-resistant HL phenotype. To test this CR versus PR outcome model in a preliminary manner, we first recategorized all 27 patients according to the proposed model and predicted the outcome correctly in 23 (85%) of 27 patients (Fig. 4). Four newly acquired HL samples were then tested in a blind fashion. One sample showed loss of 4q27 without \( BCL3 \) gains and was correctly assigned primary refractory; the remaining 3 cases were correctly designated “chemosensitive;” however, 1 “chemosensitive” patient (BK21) who initially responded to treatment suffered relapse of disease 10 years later.

**Discussion**

The identification of recurring cytogenetic and molecular alterations in HL has been thwarted by the infrequent occurrence of HRS cells in affected lesions (4). To gain further insight into the molecular genetic alterations in HL,
we used a combined LCM-WGA-aCGH approach to analyze the genomic composition of HRS cells from 27 diagnostic samples from patients with classical HL. As expected, the molecular karyotypes of the HRS cells revealed numerous CNAs with recurring gains more common than losses due to the ploidy variation associated with mononucleated Hodgkin and multinucleated Reed–Sternberg cells. To refine our analysis, we limited this study to include the most common CNAs, defined as gains and losses in more than 35% of the HL samples, and confirmed by chromosome region–specific FISH analyses in CD30⁺ HRS.

Previous studies have shown that multiple signaling pathways and transcription factors are deregulated in HRS cells including NF-κB, JAK (Janus-activated kinase)-STAT, and PI3K (phosphoinositide-3-kinase)-AKT (4, 7, 20). Interestingly, regions with the most frequent gains in this study

![Figure 2. Comparison of HL samples with benign follicular hyperplasia (BFH). A, concordant and discordant genes among RFH follicular and the CR HL–sensitive and the PR HL–resistant samples. A total of 151 genes were frequently detected in both CR HL and PR HL samples but not in the RFH samples, 81 genes were commonly detected in the CR HL, and 312 genes more frequently observed in the PR HL tumors and not in the other 2 groups. B, a second assessment of the CR and PR genes showed the differences and similarity between the sensitive (sen) and resistant (res) samples.](image_url)

![Figure 3. Calcium signaling and IL21/IL2 pathway interactions in HL. Top scoring network of interactions among the differential CNAs in CR HL versus PR HL. The interactions indicate differences in the Ca²⁺ and IL21/IL2 signaling pathways. Inset, legend functional classes of the genes noted. Solid line, direct interaction. Dotted line, indirect interaction.](image_url)
Inactivation by hypermethylation of \textit{NOTCH1} activity in HRS cells (21). In this study, gains of other cancers also indicate that \textit{SPRY1} and \textit{NELLI}, 2 receptor tyrosine kinase signaling genes (31, 32) also have tumor suppressor roles. \textit{SKP2} (F-box protein family) encodes a critical component of the ubiquitin ligase complex that regulates the G1/S transition of the cell cycle by mediating target proteins such as CDKN1B cyclin-dependent kinase inhibitor and RELB (33). Consistent with the phenotype of HRS cells, \textit{SKP2}-knockout experiments in mice show a hypoproliferative phenotype with serious cellular defects including nuclear enlargement, polyploidy, an increased number of chromosomes, and chromosomal instability (34). Two other notable losses observed in H1L were the \textit{TXNIP}, a gene required for natural killer cell maturation, and \textit{PPARGC1A}, a gene reported to antagonize NF-\kappaB activation and the inflammatory gene expression of \textit{PTGS2} (prostaglandin endoperoxide synthase-2; also known as cyclooxygenase-2 or \textit{COX2}; ref. 35).

A comparison between the HRS and normal GC molecular karyotypes showed a shared relationship with gains in 4 transcription factors associated with hematopoietic stem cells and early lymphocyte development. \textit{IRF8} is a key transcription factor that modulates the myeloid versus lymphoid lineage choice by hematopoietic stem cells via interactions with the ETS family proteins encoded by \textit{SPI1} or \textit{SPIH}. \textit{SPI1} encodes an ETS domain transcription factor (PU.1) that activates gene expression during myeloid and
B-lymphoid cell development, whereas SPIB acts as a lymphoid-specific enhancer to promote development of plasmacytoid dendritic cells or natural IFN-producing cells (36, 37). Specifically within the GC, IRE8 is part of the transcriptional network governing B-cell lineage differentiation including the transcriptional regulation of activation-induced cytidine deaminase and BCL6 (37). Finally, ICOSLG is a ligand for the T-cell–specific cell surface receptor ICOS, and the product of this gene acts as a costimulatory signal for lymphoid proliferation and cytokine secretion to mediate local tissue responses to inflammatory conditions. Of interest, ICOS is highly expressed on tonsillar T cells, which are closely associated with B cells in the apical light zone of GC (38). Taken together, the commonality of CNAs observed between GC and HRS cells substantiates a hematopoietic stem cell–derived progenitor cell relationship (4, 39).

A further comparison of the GC and aCGH HL results defined a subset of 151 CNAs consistently associated with HL. The 10 most frequent gene alterations in HL localized to 3 chromosomal regions and involved genes associated with inflammation, cell-cycle progression, and NF-κB/P3K signaling: losses of AMACR, GDNF, SKP2 at 16q13, gain of SHH/7q36, and gains of ABL1, CDK9, ENG, LCN2, PTGES, and TSC1 at 9q34. As noted earlier, loss of SKP2 appears to be critical for the HRS hypoproliferative phenotype. The short-lived protein Cdk9 (the product of the CDK9 gene) is also regulated by the SCF (SKP2) ubiquitin ligase complex, suggesting loss of SKP2 and gains of CDK9 allows for a stronger association with TRAF2 and continued NF-κB activation (40). LCN2 (lipocalin 2, previously known as NGAL) mediates inflammatory responses and has binding sites for NF-κB, the hematopoietic transcription factors GATA-1 and SPI1 (PU.1), as well as the matrix metalloproteinases (MMP), which protect them from degradation and promote tumor cell invasion and metastasis (41). A link between MMP and LCN2 in this study is intriguing, as Steidl and colleagues recently reported that overexpression of MMP11 is consistently associated with a tumor-associated macrophage expression profile and shortened survival in HL (9). Finally, gains of PTGS2 (formerly known as COX2) are thought to enhance tumor angiogenesis, suppress antitumor immunity, and possibly play an important role for cytokine-driven PTGS2 NF-κB activation (42).

Our genome-wide discrimination analyses on CNAs revealed distinct clusters that correlated closely with the favorable and unfavorable IPS in our HL patients suggesting the possibility of different chemoresponsive genomic profiles at disease presentation. To test this hypothesis, we compared the 2 distinct HL subgroups, selected 20 genes from 10 variable CNAs, and uploaded the data into IPA tools 3.0 to explore gene function and interactions. The data suggested that a key difference between the CR and PR molecular karyotypes may be opposing calcium signaling and IL21/IL2 pathway interactions within the tumor microenvironment. The CR patients showed frequent gains of 16q13, a chromosome region known to house genes that regulate T-cell trafficking or NF-κB activation (CCL22, CX3CL1, CCL17, DOK4, and IL10). HRS cells are known to secrete CCL17 and CCL22 which attracts TH2 and regulatory T cells; report higher numbers of CD4+CD25 (high) FOXP3+ regulatory T cells are reported to predict for improved survival and prognosis in non–Hodgkin lymphoma (43). Further characterization of the tumor microenvironment may provide insight on the differential regulation of regulatory T cell function and survival in HL.

In contrast, PR HL showed frequent loss of genes at 4q27, 5p15, 11p14.3, 17p12 and gains at 13q31.2 and 19q13.31. Losses of 2 cytokine genes, IL2 and IL21 within 4q27, were evident in about 50% of the PR HRS cells. IL21 exhibits potent antitumor responses, is homologous to IL2, and shares the common cytokine receptor γ chain of the IL2 receptor family (44). Although IL21 expression is usually restricted to subsets of CD4+ T cells and natural killer cells (44), two recent studies describe aberrant expression of IL21 activating STAT3 and STAT5 in HL continuous cell lines and a subset of primary HRS cells (3, 45). Moreover, Scheeren and colleagues (45) have shown that constitutive activation of STAT5 by IL21 results in activated NF-κB activity in immortalized B cells that have lost their B-cell phenotype and therefore mimic HRS cells, supporting a link between STAT5 and NF-κB in a subset of HL.

Conversely, the loss of IL21 in PR HL, as seen in our study, may prompt additional mutations in HRS cells to maintain cell survival in a cytokine-independent manner. One such complementary mutation may be gain of BCL3/RELB at 19q13.3. Previous to this report, only a rare primary cases of HL (~15%) showed BCL3 gains or translocations in HRS cells (15, 46); however, gains of BCL3 appear to be more common in HL cell lines (12). In this study, we show that BCL3 gains are common in PR HL but infrequent findings in CR HL (~50% vs. 15%, respectively). Of interest, BCL3 not only regulates the NF-κB signaling pathway by modulating the DNA-binding activity of NF-κB transcription factors involved in apoptosis and cell growth but also inhibits p53-induced apoptosis (47). Because IL21 is a potent inducer of BCL3 (48), the absence of IL21 may trigger BCL3 gene amplification as a mechanism for HRS cell survival. Interestingly, gains or overexpression of BCL3 have been associated with poor prognosis in other lymphoid malignancies including HTLV-1–induced ATL and multiple myeloma (48, 49). Finally, losses within chromosome band 17p12 could possibly result in genomic rearrangements leading to overexpression of CD68, a gene housed within the neighboring chromosome band 17p13.1. Increased numbers of CD68+ macrophages in HL have been correlated with short disease-free survival and a high likelihood of relapse post–autologous stem cell transplantation (9). Based on our aCGH HL findings, we propose HL samples without gains in 16q13 and losses of IL21/IL2 at 4q27, 11p14.3/SLC17A6 and/or 17p12 and possibly concurrent gains of BCL3 portend a chemoresistant HL phenotype. A preliminary evaluation of the model appeared to support our hypothesis and the finding of 16p13 gains in the CR samples suggests that genes housed in this region may predict for a chemosensitive HL.
phenotype. More detailed explorations of these loci are needed to identify precise and reproducible prognostic genetic markers in HL.

Even though BAC aCGH technology is robust and provides high signal-to-noise ratios when working with challenging samples, such as HRS cells, captured by LCM from FFPE tissue, there are some limitations to its use for development of new clinical prognostic assays in HL. Because aCGH does not detect inactivating mutations and our selection criteria precluded the detection of small deletions, loss of the small approximately 16-kb TNFAIP3 (or A20) at 6q23.3 was not detected in this study. We also did not observe amplification of the 42-kb REL gene within 2p16.1 as reported by others in about 30% to 40% of classical HL (5, 46); however, gains within chromosome band 19q13.3 which houses both BCL3 and RELB were observed. Most likely, our strict inclusion criteria of investigating only gains and losses observed in more than 35% of samples tested and within a 5-BAC region (>700 kb) precluded detection of the small gene gains and losses, suggesting the necessity for higher resolution microarray platforms in future studies.

Clarification of the genetic alterations and molecular mechanisms that underlie the oncogenic HRS cell are likely to improve our current understanding of HL. In this study, we identified DNA CNAs in HL by aCGH using DNA extracted from microdissected CD30+ HRS cells, showing the potential utility of this approach for defining prognostic parameters. Despite the limited sample size, this HL investigation is one of the largest to date analyzing the molecular karyotypes of well-characterized, primary diagnostic HL samples with long-term follow-up. The molecular data showed a clear relationship between CNAs of key genes associated with the NF-κB signaling pathway, the tumor microenvironment, cell-cycle regulation and apoptosis; however, the functional consequences of many of these genetic lesions and their complex interactions in HL tumorigenesis remain to be elucidated. Finally, the genetic differences observed between the chemosensitive and PR HL molecular karyotypes, in this study and others (5–7, 9), suggest that important pathogenetic and prognostic differences are present at disease onset. Given the paucity of PR HL tumor samples available for study and the rarity of HRS cells within these lesions, it is imperative that we unite our efforts for the collection of retrospective HL samples with long-term follow-up data for ongoing studies to confirm, refine, and define relevant biomarkers in PR HL. Further studies exploiting these genetic lesions and defining their role in the pathogenesis of HL will be essential in developing new targeted therapeutic approaches.

**Added note in support of our findings**

During the manuscript review process, an interesting and similar aCGH in classical HL study using laser microdissected HRS cells was published by Steidl and colleagues (50). Both studies agree that gains and losses of genes involved in the NF-κB signaling pathway are recurrent findings in classical HL. Unlike our study, Steidl and colleagues (50) report on the significance of gains and overexpression of the MDR gene ABCG1 in their treatment failure cohort, which consisted of lymph node samples collected from both treatment-naïve and treatment-exposed HL patients. We did not find a correlation between gains of ABCG1 and drug resistance; however, our population consisted only of newly diagnosed pretreatment samples and we used a slightly different method (FFPE not frozen samples). Although our study size is smaller, we only utilized samples collected at HL disease presentation (no relapsed samples) to eliminate bias associated with treatment for our comparison of chemosensitive versus PR HL. This more homogenous patient population in combination with stringent gain and loss threshold requirements makes our data equally compelling.

**Disclosure of Potential Conflicts of Interest**

M.L. Slovak is currently an employee of Quest Diagnostics. The other authors declared no potential conflicts of interest.

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**References**


Molecular Karyotypes of Hodgkin and Reed–Sternberg Cells at Disease Onset Reveal Distinct Copy Number Alterations in Chemosensitive versus Refractory Hodgkin Lymphoma

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