Classical Hodgkin Lymphoma Is Associated With Frequent Gains of 17q

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The etiology of Hodgkin lymphoma (HL) is poorly understood, and studies of the genetics of this disease have been hampered by the scarcity of the Hodgkin and Reed–Sternberg (HRS) cells within tumors. To determine whether recurrent genomic imbalances are a feature of HL, CD30-positive HRS cells were laser-microdissected from 20 classical Hodgkin lymphomas (cHLs) and four HL-derived cell lines and subjected to analyses by comparative genomic hybridization. In primary tumors, the most frequently involved chromosomal gains were 17q (70%), 2p (40%), 12q (40%), 17p (40%), 22q (35%), 9p (30%), 14q (30%), and 16p (30%), with minimal overlapping regions at 17q21, 2p23–13, 12q24, 17p13, 22q13, 9p24–23, 14q32, 16p13.3, and 16p11.2. The most frequent losses involved 13q (35%), 6q (30%), 11q (25%), and 4q (25%), with corresponding minimal overlapping regions at 13q21, 6q22, 11q22, and 4q32. Statistical analysis revealed significantly more gains of 2p and 14q in the older adult cases; loss of 13q was associated with a poor outcome. The results suggest that there is a set of recurrent chromosomal abnormalities associated with cHL and provide further evidence that cHL is genetically distinct from nodular lymphocyte predominance Hodgkin lymphoma (NLPHL). Abnormalities of 17q are infrequent in other lymphomas or NLPHL; this finding, coupled with current knowledge of gene expression in cHL, suggests that genes present on 17q may play an important role in the pathogenesis of cHL.

INTRODUCTION

Hodgkin lymphoma (HL) is the most common malignant lymphoma in young people in the Western world. HL is unusual among malignancies in that the tumor cells, the Hodgkin and Reed–Sternberg (HRS) cells, make up a small portion of the total tumor mass. The major part is a reactive infiltrate including B- and T-lymphocytes, eosinophils, plasma cells, and fibroblasts. HL is classified on the basis of both the morphology and immunophenotype of the HRS cells and the composition of the reactive component of the lesions. On the basis of distinct biological, histological, and clinical features, nodular lymphocyte predominance Hodgkin lymphoma (NLPHL) is thought to represent a distinct entity and is classified separately from the other subtypes, which are collectively known as classical HL (cHL). The World Health Organization (WHO) classification system recognizes four subtypes of cHL: lymphocyte-rich (LR), nodular sclerosis (NS), mixed-cellularity (MC), and lymphocyte-depleted (LD) (Stein et al., 2001).

The scarcity of the HRS cells within tumors, usually <1% of the total cells, combined with the difficulty of enriching these cells from biopsy specimens, has hampered studies of the molecular genetics and cytogenetics of HL. The introduction of techniques for manipulating single HRS cells, coupled with analysis by PCR, has led to significant improvement in our understanding of the biology of HL. The finding from single-cell studies that HRS cells have clonal rearrangements of immunoglobulin genes, with evidence of somatic hypermutation, suggests that HRS cells in most cases of cHL are derived from germinal center B cells (Kuppers and Rajewsky, 1998). HRS cells do not express surface immunoglobulin, and it is therefore not clear how they are able to escape apoptosis, the normal fate of germinal center B cells that lack high-affinity antibody (Rajewsky, 1996; Lam et al., 1997).

In around one third of cases in developed countries, HL is associated with the Epstein–Barr virus (EBV), and this association is believed to be causal...
HRS cells in the EBV-associated cases express the virally encoded LMP1 and LMP2 proteins, and it is postulated that this facilitates survival in germinal centers (Thorley-Lawson, 2001). Many of the epidemiological features of EBV-associated cases are distinct from those of EBV-negative cases, and these two groups may therefore represent subgroups with distinct etiologies. A higher proportion of MCHL than NSHL cases are EBV-positive, and older adults are more likely to be EBV-positive than cases in the young adult age incidence peak (Jarrett, 2002).

Cytogenetic analysis has been extremely productive in the investigation of non-Hodgkin lymphoma and leukemia, where the identification of non-random chromosomal abnormalities has led to the discovery of numerous oncogenes (Rowley, 1973; Pelicci et al., 1986; Rosenberg et al., 1991; Raimondi, 1993; Tilly et al., 1994; Caligiuri et al., 1997; Pulford et al., 1997; Dierlamm et al., 1999). A conventional cytogenetic approach to the investigation of HL has been hindered because of the small number and low mitotic index of HRS cells. Normal karyotypes have been found in almost half the cases studied, with the rest showing a variety of complex chromosomal abnormalities (Sarris et al., 1999). It is difficult to be certain that the results obtained are truly representative of the malignant cell populations.

An alternative approach to studying molecular cytogenetic abnormalities in HL is to use comparative genomic hybridization (CGH) in combination with laser microdissection (LMD). LMD allows single HRS cells to be collected, and by use of the degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) it is possible to generate sufficient DNA template from small cell numbers for CGH analysis. The CGH technique can thus be used to screen clinical specimens for genetic imbalances when only a very small amount of tumor DNA is available (Kallioniemi et al., 1992). CGH has been used extensively in the studies of both solid tumors and leukemias. Such studies not only have provided a basis for the identification of genes relevant for the pathogenesis of tumors, but also have contributed to recently developed tumor classifications (Heselmeyer et al., 1996; Joos et al., 1996; Kovacs et al., 1997; Nigro et al., 2001; Verdorfer et al., 2001; Tarkkanen and Knuutila, 2002).

This report presents our CGH analysis of 20 cases of cHL. In the study HRS cells were isolated from cytospins on the basis of both CD30 immunostaining and morphological criteria, thus ensuring that pure populations of bona fide HRS cells were selected for further analysis.

**MATERIALS AND METHODS**

**Case Selection**

Twenty cases of cHL from which viable material was available were selected. Cases included 9 male and 11 female patients, aged 14–84 years at the time of diagnosis (Table 1). All patients gave informed consent for their tissue to be used, and this study was approved by a multicenter research ethics committee. Sections from all cases were reviewed by an expert lymphoma pathologist and classified according to the WHO guidelines (Stein et al., 2001). EBV status was determined by small EBV-encoded RNA (EBER) in situ hybridization or EBV latent membrane protein 1 (LMP1) immunohistochemistry as previously described (Armstrong et al., 1998). Cases in which the HRS cells stained positively in either assay are referred to as EBV-positive cHL.

**Cell Lines**

Validity testing of the DOP-PCR-CGH was performed by use of the following: the IM9 cell line, a lymphoblastoid cell line with a female, diploid,
stable karyotype [American Type Culture Collection (ATCC), Manassas, VA]; Daudi, a Burkitt lymphoma cell line with a male, diploid, stable karyotype (ATCC); and MPE600 (Vysis, Richmond, UK), a breast cancer cell line with known CGH profile. DNA extracted from four HL-derived cell lines (L428, L1236, KM-H2, and L591) was also investigated by use of CGH.

Laser Microdissection of HRS Cells

LMD was performed on cytopsins prepared from single-cell suspensions of fresh or viably frozen lymph node biopsies. Briefly, fresh tissue samples were mechanically disrupted by use of a Medimachine (Dako, Cambridgeshire, UK) and mononuclear cells purified by density gradient centrifugation over Lymphoprep (Nycomed United Kingdom, Birmingham, UK). Aliquots of 1–5 × 10⁵ cells were spotted onto non-charged microscope slides covered in a layer of PENfoil film (PALM, Bernried, Germany) by use of a Cytospin 2 cytocentrifuge (ThermoShandon, Cheshire, UK). Slides were air-dried, and then the cells were fixed in ice-cold acetone for 5 min. Immunohistochemistry was performed by use of a CD30 monoclonal antibody, HRS-4 (Beckman Coulter, Buckinghamshire, UK), followed by a cascade of rabbit anti-mouse secondary antibody and avidin–biotin complex conjugated with alkaline phosphatase (Dako). FastRed was used as the chromogenic substrate (Dako) and 0.1% toluidine blue as the counterstain. Identification of HRS cells was based on positive staining for CD30 antigen coupled with morphological criteria (Fig. 1). Fifty single HRS cells were microdissected from each case into the caps of five PCR tubes (10 cells per cap) by use of the Leica Laser Microdissection system (Leica Microsystems, Milton Keynes, UK).

DOP-PCR

Laser-microdissected cells were lysed in 6 μl of ThermoSequenase buffer containing non-ionic detergent and proteinase K (26 mM Tris-HCl, pH 9.5, 6.5 mM MgCl₂, 0.45% Tween 20, 0.45% NP-40, and 0.25 mg/ml proteinase K) by incubation at 55°C for 1 hr. Proteinase K was subsequently inactivated by incubation at 95°C for 10 min. DOP-PCR was performed by use of a GeneAmp PCR System 2400 or 9700 (Applied Biosystems, Warrington, UK) in two steps according to a published protocol (Huang et al., 2000). In step I, DNA was amplified in a 10-μl reaction volume containing 200 μM of each dNTP, 1 μM UNI-primer (Telenius et al., 1992), 4 units of ThermoSequenase DNA polymerase (Pharmacia Biotech, Buckinghamshire, UK), and 1 × ThermoSequenase reaction buffer (26 mM Tris-HCl, pH 9.5; 6.5 mM MgCl₂). Thermal cycling conditions consisted of 3 min at 95°C, followed by four cycles of 1 min at 94°C, 1 min at 25°C, a 3-min transition from 25 to 74°C, a 2-min extension at 74°C, and a final extension of 10 min at 74°C. In step II, the reaction volume was increased to 50 μl by the addition of 40 μl of a mastermix containing 160 μM of each dNTP, 1.2 μM UNI-primer, 5 units AmpliTaq DNA polymerase (Applied Biosystems), and 1 × PCR buffer (10 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM MgCl₂; and 0.001% gelatin; Applied Biosystems). Thermal cycling conditions were 3 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, a 2-min extension at 72°C, and a final extension of 10 min. DOP-PCR reactions including 60
pg of normal male and female DNA (Promega, Southampton, UK) were used to generate reference control DNA for CGH. Replicates of 10 IM9 cells and 10 Daudi cells obtained by LMD and aliquots of 60 pg of MPE600 DNA were subjected to DOP-PCR for use in CGH validity testing experiments.

**DNA Labeling and CGH Analysis**

Reaction products from the five DOP-PCRs from each cHL case were pooled and ethanol-precipitated before labeling. The quantity of DOP-PCR products was estimated by use of a GeneQuant spectrophotometer (Pharmacia Biotech) and by running one tenth of the DOP-PCR products on a 1% agarose gel. Approximately 1.5 μg of DOP-PCR product from test and reference samples was labeled with SpectrumGreen-dUTP or SpectrumRed-dUTP, respectively (Vysis). Nick translation was carried out by use of an established protocol incorporating DNA Polymerase I and DNase I (Invitrogen, Paisley, UK).

Approximately 800–1,000 ng of each labeled DNA were combined with 60 μg of human COT-1 DNA (Invitrogen) and hybridized to normal male metaphase target slides (Vysis) for 4 days at 37°C. After hybridization, the slides were washed in commercially available wash buffers (Vysis), and the chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vysis). Digital images were captured by use of a cooled charge-coupled device camera (Photometrics, Ottobrunn, Germany) connected to a Zeiss Axioskop fluorescence microscope (Carl Zeiss, UK). Images were analyzed with Quips CGH software (Vysis). Results from five to 10 optimal target metaphases were combined to produce the final CGH result in each case. Chromosome 19 centromeric and telomeric regions were excluded from the analysis because of possible non-uniform or incomplete blocking of repeated sequences in these regions (Kallioniemi et al., 1994). Because the tumor specimens and reference DNA were not sex-matched, the X and Y chromosomes were also excluded.

**Analysis of Results**

Results were analyzed by visual inspection of an ideogram containing results from all cases, and by recording amplifications and deletions of both chromosome arms. Recurrent imbalances of chromosomal arms were identified as gains affecting ≥30% of cases or losses affecting ≥25% of cases. A lower threshold was applied to losses because CGH is less sensitive in the detection of deletions. The total number and presence or absence of recurrent imbalances were analyzed with respect to age group, sex, histological subtype, outcome, and EBV status of tumors. Age groups were defined as those <35 years old, 35–55 years old, and >55 years old. Poor outcome was defined as relapse or death. Statistical testing used the two sample t-test, Fisher’s exact test (two-tailed), and Kruskal–Wallis test. Analyses were implemented by use of SPSS software (SPSS, Woking, UK).

**RESULTS**

In validity testing experiments, all of the expected abnormalities were identified by use of DOP-PCR products from the MPE600 cell line, whereas normal profiles were obtained by use of laser-microdissected IM9 and Daudi cells. Optimization experiments that used material obtained by LMD revealed that amplification of five replicates of 10 HRS cells gave a better yield and size range of DOP-PCR products than amplification of 50 HRS cells in a single tube (data not shown). Satisfactory CGH results were obtained for the 20 cHL cases analyzed by use of this strategy, and results are presented in Figure 2 and Table 2.

Gains affecting ≥30% and losses affecting ≥25% of the cases were identified. Twelve chromosomal arms were involved; only one case did not have any abnormality affecting these arms. The most frequent gains were on chromosome 17, with gains on 17q and 17p detected in 14/20 and 8/20 cases, respectively. The most commonly over-represented regions were 17q21 and 17p13. Other frequent gains involved 2p (40%), 12q (40%), 22q (35%), 9p (30%), 14q (30%), and 16p (30%), with minimal overlapping regions at 2p23–13, 12q24, 22q13, 9p24–23, 14q32, 16p13.3, and 16p11.2. The most frequent losses involved 13q (35%), 6q (30%), 11q (25%), and 4q (25%), with corresponding minimal overlapping regions at 13q21, 6q22, 11q22, and 4q32. Analysis of individual chromosome arms revealed significantly more gains of 2p and 14q in the older adult cases (P = 0.038 and P = 0.022, respectively); losses of 13q were associated with a poorer outcome (P = 0.049). The number of chromosomal imbalances ranged from 0 to 21, with a mean of 10. Amplifications were more frequent than deletions, with a mean value of seven gains and three losses.

Results from CGH analysis of the EBV-negative, HL-derived cell lines L428, L1236, and KMH2 showed resemblance to results from analysis of primary HRS cells (Table 3). Shared abnormalities included gains of 2p, 12q, 17p, 16p, and 14q and...
Figure 2. CGH findings in 20 cases of classical Hodgkin lymphomas (cHL). Bars to the left of the ideograms indicate regions of copy number loss and bars to the right of the ideograms indicate regions of copy number gain. Chromosome 19 was excluded because of false positives in negative controls, and the X and Y chromosomes were excluded because of non-sex-matched reference and tumor DNA.
losses of 13q, 6q, 4q, and 11q. In contrast the EBV-positive HL-cell line L591 showed a different CGH pattern with fewer abnormalities; these included gains in 7q and 9p and losses of 8p, 9q, and 22q.

**DISCUSSION**

Analysis of 20 cases of cHL by use of CGH revealed recurrent chromosomal imbalances. Imbalances on 12 chromosomal arms were considered recurrent because these arms were affected in...
30% of cases for gains and 25% of cases for losses (Figs. 3A and 4A). The most frequent abnormality was gain on 17q (70%). Other frequent gains involved 2p (40%), 12q (40%), 17p (40%), 22q (35%), 9p (30%), 14q (30%), and 16p (30%). The most frequent losses involved 13q (35%), 6q (30%), 11q (25%), and 4q (25%).

Two other laboratories have investigated chromosomal imbalances in cHL by use of CGH (Ohshima et al., 1999; Joos et al., 2000, 2002). Because of the difficulty of working with cHL, different techniques were used to obtain HRS cells, and the results of the studies were not consistent. In the present study, HRS cells were obtained by LMD from cytospin...
preparations that had been immunostained by use of CD30 monoclonal antibodies. This method of collection of HRS cells offers advantages over previous methods used in CGH experiments in that single HRS cells were individually selected through the use of stringent criteria, and the use of cytospins prepared from viable material ensured that complete cells were captured; hence the robust nature of this study. Ohshima et al. (1999) sorted CD30-positive giant cells by flow cytometry by use of an automatic cell-deposition unit. Most of the abnormalities reported here were not detected either in the present study or in that of Joos et al. (2000). In contrast, the data presented here are largely similar to those of Joos et al.
on 2p and 14q in the older adult cases (ther testing. There were significantly more gains of cases analyzed was small and statistical power limited, these analyses provide hypotheses for further studies.

The chromosomal imbalances detected in cHL are different from those in most other lymphomas (Avet-Loiseau et al., 1997a; Barth et al., 1998, 2001; Aalto et al., 1999; Siu et al., 1999; Tsukasaki et al., 2001; Allen et al., 2002), with the exception of primary mediastinal B-cell lymphoma (PMBCL). PMBCL is the only NHL that shares frequent abnormalities in previous cytogenetic studies of cHL (Avet-Loiseau et al., 1997a; Barth et al., 1998, 2001; Aalto et al., 1999; Siu et al., 1999; Tsukasaki et al., 2001; Allen et al., 2002; Palanisamy et al., 2002). Although the retinoblastoma (RB1) gene is located on 13q14, this tumor suppressor gene does not appear to be the target of deletions in cHL (Weiss, 1995; Guenova et al., 1999; Kanavaros et al., 2000) and other lymphomas (Liu et al., 1995; Stilgenbauer et al., 1998). Expression of the RB protein has been found in most cases, suggesting the presence of another tumor suppressor gene on 13q13–q21. Loss of 6q has been associated with a poor prognosis in diffuse large B-cell lymphoma (Harada et al., 2001), follicular lymphoma (Tilly et al., 1994), and acute lymphoblastic leukemia (Merup et al., 1998), but as yet no tumor suppressor gene has been identified in the 6q21–q27 region (Offit et al., 1993; Haupt-schein et al., 1998).

With the exception of L1236, HL-derived cell lines have an uncertain relationship to the tumors from which they were derived (Wolf et al., 1996). Results of CGH analysis of the EBV-negative HL-derived cell lines showed a marked resemblance to the results from primary HRS cells, including gains of 2p, 12q, 15q, 17p, 16p, and 14q and losses of 13q, 6q, 4q, and 11q. In contrast, the EBV-positive–derived cell line L591 showed a very different CGH pattern with few abnormalities. This observation in L591 may relate to the EBV status of the cells or suggest a non-HRS cell origin (Drexler, 1993).
In summary, this CGH study has identified a set of recurrent chromosomal abnormalities associated with cHL, with gain of 17q being the most frequent abnormality in this series of cHL. Abnormalities of 17q are infrequent in NHL or NLPHL; this finding, coupled with current knowledge of gene expression in cHL, suggests that genes present on 17q may play an important role in the pathogenesis of cHL.

ACKNOWLEDGMENTS

We thank all of the pathologists and clinicians who contributed samples to this study, in particular Dr. Mark Vickers. This work was supported in part by a CSO Research Training Fellowship (to D.C.) from the Scottish Executive.

REFERENCES


FREQUENT GAINS OF 17q IN CLASSICAL HODGKIN LYMPHOMA


