Detection of genomic imbalances in microdissected Hodgkin and Reed-Sternberg cells of classical Hodgkin's lymphoma by array-based comparative genomic hybridization

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ABSTRACT

Background

Cytogenetic analysis of classical Hodgkin's lymphoma is limited by the low content of the neoplastic Hodgkin-Reed-Sternberg cells in the affected tissues. However, available cytogenetic data point to an extreme karyotype complexity. To obtain insights into chromosomal imbalances in classical Hodgkin's lymphoma, we applied array-based comparative genomic hybridization (array comparative genomic hybridization) using DNA from microdissected Hodgkin-Reed-Sternberg cells.

Design and Methods

To avoid biases introduced by DNA amplification for array comparative genomic hybridization, cHL cases rich in Hodgkin-Reed-Sternberg cells were selected. DNA obtained from approximately 100,000 microdissected Hodgkin-Reed-Sternberg cells of each of ten classical Hodgkin's lymphoma cases was hybridized onto commercial 105 K oligonucleotide comparative genomic hybridization microarrays. Selected imbalances were confirmed by interphase cytogenetics and quantitative polymerase chain reaction analysis and further studied in an independent series of classical Hodgkin's lymphoma.

Results

Gains identified in at least five cHL affected 2p12-16, 5q15-23, 6p22, 8q13, 8q24, 9p21-24, 9q34, 12q13-14, 17q12, 19p13, 19q13 and 20q11 whereas losses recurrent in at least five cases involved Xp21, 6q23-24 and 13q22. Copy number changes of selected genes and a small deletion (156 kb) of the *CDKN2B* (p15) gene were confirmed by interphase cytogenetics and polymerase chain reaction analysis, respectively. Several gained regions included genes constitutively expressed in cHL. Among these, gains of *STAT6* (12q13), *NOTCH1* (9q34) and *JUNB* (19p13) were present in additional cHL with the usual low Hodgkin-Reed-Sternberg cell content.

Conclusions

The present study demonstrates that array comparative genomic hybridization of microdissected Hodgkin-Reed-Sternberg cells is suitable for identifying and characterizing chromosomal imbalances. Regions affected by genomic changes in Hodgkin-Reed-Sternberg cells recurrently include genes constitutively expressed in cHL.

Key words: Hodgkin's lymphoma, array comparative genomic hybridization, microdissection.

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The online version of this article contains a supplemental appendix.

Introduction

Classical Hodgkin´s lymphoma (cHL) is one of the most common malignant lymphomas in the western world. Although it can be cured in almost 80% of the cases, patients frequently develop secondary malignancies due to chemotherapy and radiotherapy. Hodgkin-Reed-Sternberg (HRS) cells, the tumor cells in cHL,[1,2] represent 1% or less of the cHL typical infiltrate, which consists mainly of lymphohistiocytic cells.[3] Low tumor cell content in the tissues and poor chromosome morphology of metaphases derived from HRS cells have largely prevented classical cytogenetic analysis of cHL. Nevertheless, it has been possible to characterize some HRS cells by classical cytogenetics and they have been shown to have complex and instable hyperploid karyotypes with multiple marker chromosomes. The most recurrent chromosomal breakpoints targeted chromosome arms 1p, 6q, 7q, 11q, 12q and 14q[4] whereas recurrent gains were frequently observed on chromosomes 2, 5, 9 and 12, and losses on chromosomes 13, 21 and Y.[5-7]

Taking advantage of the fact that HRS cells express CD30, the combination of anti-CD30 immunofluorescence with interphase fluorescence *in situ* hybridization (FISH) (FICTION technique) confirmed that HRS cells in cHL show numerical abnormalities and intraclonal variability.[8] Additionally, chromosomal translocations affecting the immunoglobulin loci, which are characteristic of non-Hodgkin's B-cell lymphomas, have also been described in 20% of cHL cases, involving oncogenes such as *BCL6, MYC, BCL3* or *REL* as translocation partners in a few cases. However, no recurrent HRS cell-characteristic translocation has yet been identified.[9,10] Although FISH and FICTION are suitable methods for the determination of chromosomal copy numbers and translocations, they are only applicable to selected candidate loci.

Comparative genomic hybridization (CGH), instead, is a genome-wide screening method for the detection of copy number aberrations which is independent of the presence of metaphases in tumor cells.[11] In classical metaphase CGH, tumor and reference DNA are differentially labeled and co-hybridized onto spreads of metaphase chromosomes from healthy donors, providing a resolution of approximately 2 Mb for high-level amplifications and 10-20 Mb for deletions.[12] Metaphase CGH in primary cHL has been performed using degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR)-amplified DNA from HRS cells.[13,14] These studies led to the identification of recurrent imbalances in cHL, such as chromosomal gains involving 2p, 9p, 12q and 17q and losses affecting 13q.

To improve the resolution and specificity of CGH, different types of arrays (e.g. bacterial artificial chromosome [BAC] arrays, cDNA arrays, oligonucleotide arrays) have been developed.[15-18] Array CGH analyses of cHL have so far been focused on a few well-characterized cHL cell lines,[19] which were all established from relapsed cases after treatment.

In order to investigate primary HRS cells from lymph nodes by CGH, the cells have to be microdissected from frozen tissue sections and the DNA isolated from the few HRS cells usually has to be amplified using methods such as DOP-PCR, multiple-strand displacement amplification and linker-mediated PCR.[14,20-22] These methods introduce a certain bias for the detection of copy number changes because not all genomic regions are linearly amplified.[23] While such amplification biases may be irrelevant for metaphase CGH,[14] they might represent a severe problem for high resolution array CGH,[20,22] particularly if oligonucleotide arrays are applied. In this study, therefore, we analyzed cHL cases with a high content of HRS cells by array CGH without prior DNA amplification.

Design and Methods

Cases and microdissection

Lymph node samples from 12 patients with cHL, rich in HRS cells, were included in the study. According to morphological and immunohistochemical criteria seven cases were classified as lymphocyte-depleted cHL and five cases as nodular sclerosis grade II, respectively.[24] HRS cells were partly lying in clusters with a HRS cell content of at least 70% in microdissected areas. HRS cells showed the following antigen expression pattern: CD30 positive, ALK-1 negative and positive for at least one of the markers EBER, CD15 and PAX5 (*Online Supplementary Table S1*), hence excluding the differential diagnosis of anaplastic large cell lymphoma. For interphase cytogenetic analysis, cytogenetic suspensions from 28 cHL cases with the usual low HRS cell content were used. Informed consent was obtained in accordance with the Declaration of Helsinki and the local ethics committees approved the study.

For microdissection, 5 µm cryosections were mounted on membrane-covered slides (PALM, Bernried, Germany), stained with CD30 (Clone BerH2, DAKO, Glostrup, Denmark, Super Sensitive Link-Label IHC Detection System BIOGENEX, San Ramon, USA), and counterstained with hematoxylin. Approximately 100,000 HRS cells per case were microdissected with an ultraviolet laser (PALM microdissection system, Zeiss Axiovert 200M microscope) in areas of 100-500 HRS cells lying in clusters.

Array comparative genomic hybridization

DNA from microdissected HRS cells was extracted using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. Commercially available sex-matched pooled DNA from healthy donors (Promega, Madison, USA) was used as reference DNA. A total of 200-500 ng DNA per case was processed according to the Agilent oligonucleotide array-based CGH protocol (Version 4.0) and hybridized onto Agilent 105 K oligonucleotide arrays (Agilent Technologies, Santa Clara, USA). Scanning was performed using an Agilent Scanner (G2565BA(G25053)). Signal intensities from the generated tif images were measured and evaluated with Feature Extraction v9.5 and CGH Analytics v3.4.27, respectively (Agilent Technologies). The quality of the array hybridizations

was determined by calculating the derivative log ratio spread (DLRS) with the Agilent CGH Analytics software. This parameter reflects the spread of log2-ratio differences between consecutive probes along all chromosomes. For samples lacking chromosomal aberrations the DLRS should be approximately 0.2-0.3 (CGH Analytics user manual). Considering that HRS cells show large numbers of chromosomal imbalances and that DNA is extracted from laser-microdissected cells, cases with a DLRS above 0.8 were classified as not evaluable. Gains and losses were defined by use of the Aberration Detection Method-2 (ADM-2) algorithm of the CGH Analytics software with a threshold of 6.0.[25] Significant chromosomal gains detected by the ADM-2 algorithm with an average log2-ratio exceeding 0.8 were classified as amplifications. Copy number variations/polymorphisms were identified with a database integrated in the Agilent CGH Analytics software and excluded from further analyses. Candidate genes in gained/lost regions were selected on the basis of a database of cancer genes.[26]

FISH and FICTION

The genes *REL* (2p16.1), *CHD1* (5q21.1), *TNFAIP3/A20* (6q23.3), *TEK* (9p21.2), *NOTCH1* (9q34.3) and *CDK4* (12q14.1) as well as the centromere of chromosome 6 (CEP6, Abbott, Downers Grove, USA) were further investigated by FICTION for confirmation purposes in two of the primary cHL cases analyzed by array CGH, as described previously.[27] Further FISH analyses of *NOTCH1, STAT6* and *JUNB* were performed using routine protocols in an independent series of cytogenetic suspensions of 11, 21 and 7 primary cHL cases with normal HRS cell content.[9,28,29] BAC clones used to design FISH probes for the genes listed above are shown in *Online Supplementary Table S2*. Considering that HRS cells are usually hyperploid, an estimation of the ploidy level was obtained as the mean value of copy numbers from 12 different loci (*data not shown*).[28,30] Copy numbers from FISH analyses of *NOTCH1* and *STAT6* were adjusted to the estimated ploidy levels of the cHL cases. The genomic status was then determined according to the following criteria: ratios of gene/ploidy level lower than 0.7 were considered as genomic loss, ratios between 0.7 and 1.3 as balanced, ratios higher than 1.3 and lower than 2 as genomic gains, and ratios above 2 as genomic amplifications.[28, 30] For six of seven cHL cases studied for *JUNB* the ploidy levels were not available. In those cases, the *JUNB* genomic status was determined using the following criteria: signal copy numbers below 5 were defined as balanced (considering the characteristic tri- to tetraploidy of HRS cells), between 5 and 8 gained, and above 8 amplified.[31]

Quantitative PCR with genomic DNA

To confirm a deletion of *CDKN2B* (p15) detected in cHL case 3, quantitative multiplex seminested two-round PCR for exon 1 and exon 2 of *CDKN2B* were performed (linear positions of 21,998,857 - 21,999,001 Mb and 21,995,193 – 21,995,368 Mb on chromosome 9, UCSC Genome Browser on Human-May 2004 Assembly, PCR conditions described in the *Online Supplementary data*). PCR were carried out on sets of 100 cells each of microdissected HRS cells and reactive lymphocytes. The first round of PCR was conducted for each exon of *CDKN2B* as a multiplex reaction with *TIE1* as the reference gene. *TIE1* did not show any genomic imbalance in array CGH data and PCR primers were available from a previous study. The second round of PCR for *CDKN2B* and *TIE1* was performed as quantitative realtime-PCR in duplicates. Fold changes were calculated using the ΔΔCt-method. PCR products were sequenced, validating that the correct PCR product had been amplified.

Results

Detection of genomic imbalances in primary Hodgkin-Reed-Sternberg cells by array comparative genomic hybridization

To identify genomic aberrations at a genome-wide level in primary HRS cells, DNA from approximately 100,000 microdissected HRS cells from 12 primary cHL cases with at least 70% HRS cells in microdissected areas was hybridized to Agilent 105 K Oligonucleotide CGH arrays.

Two cases showed low quality array CGH data with a DLRS above 0.8, likely due to low quality of the starting material, and were thus excluded from further analysis. The remaining ten cases showed DLRS ranging from 0.11 to 0.78 with a median value of 0.22. Following the criteria described in the Design and Methods section, we detected 3 to 46 gains in these ten cHL cases with an average of 18.2 gains per case and an average size of approximately 41.4 Mb as well as 2 to 22 losses with an average of 11.4 losses per case and an average size of approximately 59.2 Mb (all averages as means).

The most frequent recurrent gains were detected in chromosomal regions 2p12-16, 5q15-23, 6p22, 17q12 and 19p13 (seven each of ten cases), 8q13, 8q24, 9p21-24, 9q34, 12q13-14, 19q13 and 20q11 (five of ten cases), 2p23, 5q12, 11q13, 17p13, 17q25, 21q11-21 and 22q11-12 (four of ten cases), and 7q21, 12q15-21, 16p13, 18q22, 20p13, 21q22 and Xq24-26 (three of ten cases) (Table 1). Distinct genomic amplifications were observed in single cases only (*Online Supplementary Table S3*).

Losses were observed less frequently than gains (Table 2) and occurred at Xp21 (eight of ten cases), 6q23-24 (six of ten cases), 13q22 (five of ten cases), 13q12-13, 15p13-q14, 15q14, 15q24, 16p13 and 17p13 (four of ten cases), and 4q, 6q12-15, 7q11, 8p23-12, 9q34, 16p13, 20q13 and 22p13-q12 (three of ten cases). The smallest deletion detected was the 156 kb deletion in 9p21 (21.9-22.1 Mb linear position on chromosome 9, average log2-ratio = -1.01) which included *CDKN2B* (p15) and partly *CDKN2A* (p16) (case 3) (Figure 1). Case 4 also showed a loss of the same region, but the deletion was larger (21.7-22.4 Mb) and not so pronounced (average log2-ratio=-0.2).

List of candidate genes and microRNA located in chromosomal regions gained or lost in array CGH data of HRS cells are given in Tables 1 and 2.

Confirmation of array results

A total of seven chromosomal regions (including the genes *REL, CHD1, TNFAIP3/A20, TEK, NOTCH1* and *CDK4* as well as the centromere of chromosome 6) were selected for *FICTION* studies to validate the array CGH results in two of the cHL cases. As shown in Table 3 and Figure 2, the results obtained by array CGH and FICTION showed a highly significant positive correlation. The correlation coefficient in case 5 was 0.92 (p=0.003) whereas in case 3 it was 0.89 (p=0.007). In case 5, a balanced genomic status determined by array CGH data was associated with five copies of a gene in FICTION. Genomic deletions in array CGH data were associated with four copies in FICTION and gains in array CGH data with more than five copies in FICTION. The *REL* gene was amplified according to array CGH data (log2-ratio = 0.93) and displayed a median copy number of ten in FICTION analysis. In case 3, a balanced genomic status determined by array CGH data was associated with three copies of a gene in FICTION, whereas a genomic deletion according to array CGH data correlated with two to three copies in FICTION

Table 1. Recurrent gains in primary Hodgkin-Reed-Sternberg cells.

Chromosome region	Linear position (Mb)[a]	Number of cases	Number of genes[b]	Candidate genes[c]	miRNA[d]
2p12-16	60-69	7	42	REL, BCL11A	-
5q15-23	96-125	7	64		-
6p22	31-32	7	25	TNF	-
17q12	27.8-29.3	7	7		-
19p13	8.2-15.1	7	161	JUNB, LYL1	hsa-mir-199a-1 hsa-mir-27a hsa-mir-24-2 hsa-mir-23a hsa-mir-181c hsa-mir-181d
8q13	67.1-67.2	5	3		-
8q24	145-146	5	46	RECQL4	-
9p21-24	0-29	5	70	JAK2, MLLT3, PSIP2	hsa-mir-101-2 hsa-mir-491 hsa-mir-31
9q34	134-137	5	50	CARD9, NOTCH1, TRAF2	hsa-mir-126
12q13-14	45-56.4	5	73	CDK4, GLI1, STAT6, ERBB3, ATF1, DDIT3, HOXC13	hsa-mir-196-a2 hsa-mir-148b
19q13	49-56	5	70	BCL3, RELB, ERCC2	hsa-mir-150
20q11	28.1-31.8	5	39	E2F1	-
2p23	24.6-25.9	4	8		hsa-mir-1301
5q12	50-53	4	11		-
11q13	64.8-75	4	162	RELA, CCND1, NUMA1, PRO1073	hsa-mir-612 hsa-mir-548 hsa-mir-139 hsa-mir-326
17p13	0-9.1	4	159		hsa-mir-132 hsa-mir-212 hsa-mir-1253 hsa-mir-195 hsa-mir-497 hsa-mir-324
17q25	76.8-77.8	4	20		-
21q11-21	14.5-29.9	4	26		-
22q11-12	17-28.4	4		EWSR1, NF2, PNUTL1, SMARCB1	hsa-mir-185 hsa-mir-1306 hsa-mir-1286 hsa-mir-649
7q21	80-82	3	4		-
12q15-21	70-82	3	29		hsa-mir-1252 hsa-mir-617 hsa-mir-618
16p13	0-10	3		CREBBP, TSC2	hsa-mir-662 hsa-mir-1225 hsa-mir-940
18q22	59.5-67.3	3	13		-
20p13	0-4	3	57		hsa-mir-1292 hsa-mir-103-2
21q22	32.7-46.2	3	90		hsa-mir-802 hsa-mir-1277 hsa-mir-766 hsa-mir-220a hsa-mir-363 hsa-mir-92a-2 hsa-mir-19b-2 hsa-mir-20b hsa-mir-18b hsa-mir-106a hsa-mir-450b hsa-mir-450a hsa-mir-542 hsa-mir-503 hsa-mir-934
Xq24-26	117-147	3	93	GPC3, SEPT6	hsa-mir-424 hsa-mir-504 hsa-mir-505 hsa-mir-320d-2 hsa-mir-890 hsa-mir-888 hsa-mir-892a/b hsa-mir-891a/b hsa-mir-513 hsa-mir-506 hsa-mir-507 hsa-mir-508 hsa-mir-509 hsa-mir-510 hsa-mir-514

(continue in next column)

Minimal overlapping regions recurrently gained in at least three of ten primary cHL cases. The statistical algorithm applied is ADM-2 with a threshold of 6.0. [a]Linear position on the chromosome in Mb, [b]number of genes located in gained regions, both based on UCSC Genome Browser on Human - May 2004 Assembly. [c]Candidate genes were selected on the basis of a database of known cancer genes.[26] [d]miRNA annotation, based on UCSC Genome Browser on Human - May 2004 and http://microrna.sanger.ac.uk. Twelve gains (5q12, 5q15-23, 6p22, 7q21, 8q13, 8q24, 11q13, 18q22, 19p13, 19q13, 21q11-21 and 21q22) were previously not identified in the study by Joos et al.[14]

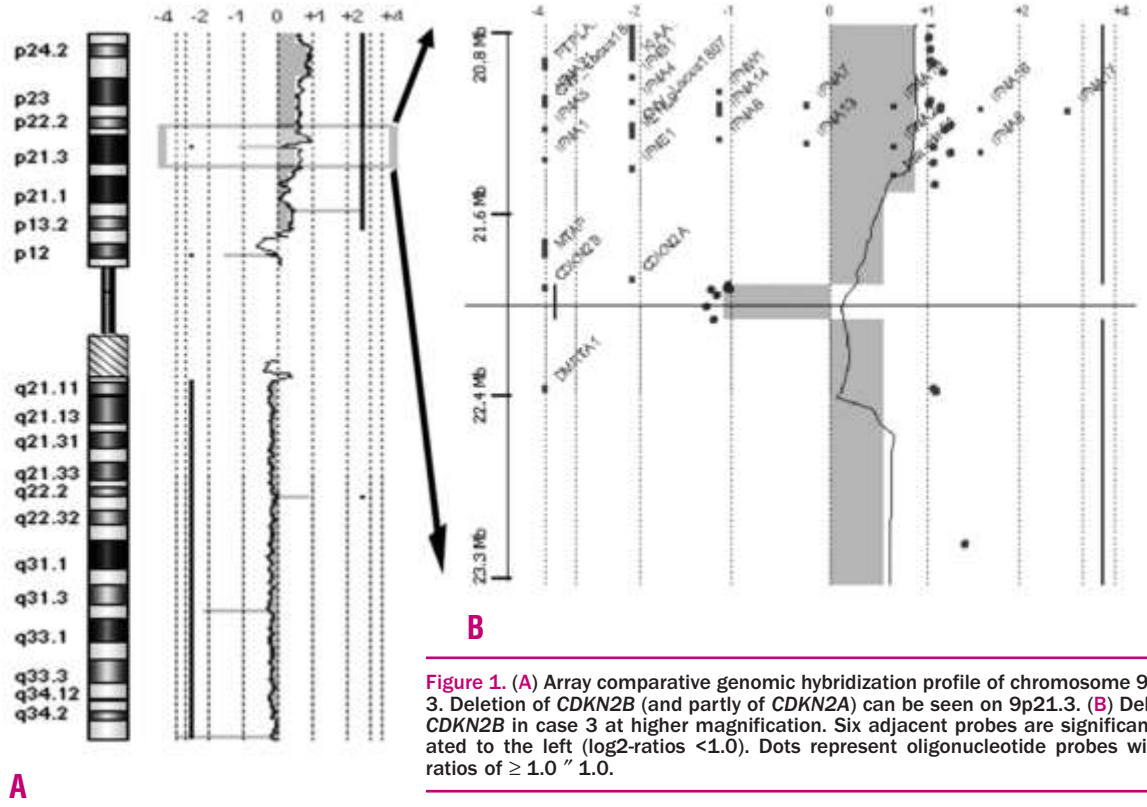

Figure 1. (A) Array comparative genomic hybridization profile of chromosome 9 of case 3. Deletion of *CDKN2B* (and partly of *CDKN2A*) can be seen on 9p21.3. (B) Deletion of *CDKN2B* in case 3 at higher magnification. Six adjacent probes are significantly deviated to the left (log2-ratios <1.0). Dots represent oligonucleotide probes with log2-ratios of ≥ 1.0 ″ 1.0.

and chromosomal gains in array CGH data with more than three copies in FICTION (Table 3, Figure 2). Thus, the two cases used for confirmation purposes displayed different ploidy levels, i.e. case 5 was approximately pentaploid whereas case 3 was approximately triploid.

In order to confirm the 156 kb long deletion targeting *CDKN2B*, quantitative multiplex realtime PCR was applied. Compared to reactive lymphocytes from the same case, HRS cells of case 3 showed 6- and 9-fold reduced amounts of PCR products for exon 1 and 2 of *CDKN2B*, respectively, relative to the control gene *TIE1* (*data not shown*). This suggests a loss of both alleles of *CDKN2B* in the HRS cells and confirms the array CGH results.

FISH analysis of cHL cases with typical low Hodgkin-Reed-Sternberg cell numbers for genomic aberrations detected by array CGH in cases with high numbers of Hodgkin-Reed-Sternberg cells

cHL cases with a content of 70% of HRS cells in selected areas are rare. To assess how often gains found by array CGH in these rare cases appear in a random selection of primary cHL cases with the typical low HRS cell content, some genes of interest were chosen (*NOTCH1* (9q), *STAT6* (12q), *JUNB* (19p)) and investigated by FISH in a new series of primary cHL cases (Table 4).

NOTCH1, located in 9q34, was gained in five of ten cases according to array CGH. By FISH, two of 11 independent primary cHL cases showed chromosomal gains, one of them a high-level genomic amplification (Table 4). *JUNB*, located in 19p13, was gained in seven

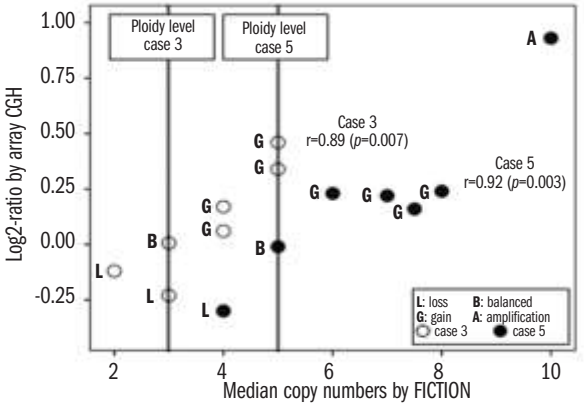

Figure 2. Scatter plot of the results obtained by array comparative genomic hybridization and FICTION in cases 3 and 5 showing a highly significant correlation between log2-ratio of array comparative genomic hybridization data (y-axis) and copy number changes determined by FICTION (x-axis).

of ten primary cHL cases analyzed by array CGH. By FISH, gains were detected in two of seven independent primary cHL cases. *STAT6*, located in 12q13, was gained in five of ten primary cHL cases analyzed by array CGH. In a series of 21 independent primary cHL cases investigated by FISH, seven cases (30%) with gains of *STAT6* were identified (Table 4). Thus, all three gains/amplifications found in HRS cell-rich cHL cases could also be detected as recurrent events in typical cHL cases with a normal HRS cell content.

Discussion

Validity of array comparative genomic hybridization results

In the present study, array CGH was performed on microdissected HRS cells from 12 cases using small amounts of DNA as starting material without any amplification of DNA. According to quality control functions of the software used for analysis, ten of 12 hybridizations were suitable for further analysis. A highly significant correlation was observed between log2-ratios in array CGH data and copy number changes of selected loci as detected by FICTION (Table 3, Figure 2), which demonstrates the validity of array CGH for detecting chromosomal imbalances in microdissected HRS cells. However, limitations of array CGH remain that on the one hand ploidy levels cannot be determined and, on the other hand, that array CGH only provides information on average copy number changes and does not allow detection of intraclonal variability, which is a characteristic feature of the chromosomally instable HRS cells in cHL.[32] This phenomenon was observed by FICTION in this study, in which varying numbers of hybridization signals in HRS cells could be demonstrated (Table 3).

Comparison between array comparative genomic hybridization and metaphase CGH to detect copy number changes in Hodgkin-Reed-Sternberg cells

With the 105 K oligonucleotide CGH arrays, overall more gains and losses per case were detected than by metaphase CGH in a previous study by Joos *et al*.[14] On average 18.2 gains per case were observed by array CGH compared to 3.9 gains by metaphase CGH and 11.4 losses per case by array CGH versus 1.1 losses per case by metaphase CGH. Most, but not all, of the previously known gained and lost regions of primary HRS cells[14] could be confirmed and further delimited by array CGH. For example, gains of chromosome 2p were observed in 54% of all cHL and in 88% of nodular sclerosing cHL by metaphase CGH.[14] The latter percentage is close to the number of gains of chromosome 2p observed in this study (seven of ten cases), in which mainly nodular sclerosing cHL cases were investigated. In addition, several new regions with recurrent genomic imbalances could be identified by array CGH (Tables 1 and 2), e.g. gains of chromosomes 5q12, 5q15-23, 6p22 7q21, 8q13, 8q24, 11q13, 18q22, 19p13, 19q13, 21q11-21 and 21q22 as well as losses of chromosomes 7q11, 8p23-12, 9q34, 15p13-q14, 15q24, 16p13, 20q13 and 22p13-q12. The smallest aberrations detected in the previous study[14] by metaphase CGH referred to single

Table 2. Recurrent losses in primary Hodgkin-Reed-Sternberg cells.

Chromosome	*Linear position (Mb)*[a]	*Number of cases*	*Number of genes*[b]	*Candidate genes*[c]	*miRNA*[d]
Xp21	25-34	8	13		hsa-mir-548f-5
6q23-24	134-142	6	27	*TNFAIP3, PERP*	–
13q22	71-83	5	16		–
13q12-13	25.4-38.5	4	54	*BRCA2, CDX2, FLT3*	–
15p13-q14	18-35	4	42		hsa-mir-1268 hsa-mir-211 hsa-mir-1233
15q14	36.5-43.7	4	77	AF15Q14	–
15q24	75.2-75.6	4	1		–
16p13	15-16.5	4	6	MYH11	hsa-mir-484
17p13	7.1-7.6	4	29	TP53	–
4q	124-191	3			
6q12-15	65-88.9	3	48		hsa-mir-30c-2 hsa-mir-30a
7q11	55.5-68.3	3	22	SBDS	-
8p23-12	0-33.5	3			hsa-mir-596 hsa-mir-548i-3 hsa-mir-597 hsa-mir-124-1 hsa-mir-1322 hsa-mir-598 hsa-mir-383 hsa-mir-383 hsa-mir-320a hsa-mir-548h-4
9q34	129-130	3	15		hsa-mir-199b hsa-mir-219-2
16p13	10.9-12	3	11	SOCS1	-
20q13	47.4-48.7	3	8		- hsa-mir-648 hsa-mir-185 hsa-mir-1306 hsa-mir-1286
22p13-q12	0-32	3			hsa-mir-649 hsa-mir-301b hsa-mir-130b hsa-mir-650 hsa-mir-548j

Minimal overlapping regions recurrently lost in at least three of ten primary cHL cases. The statistical algorithm applied is ADM-2 with a threshold of 6.0. [a]Linear position on the chromosome in Mb and [b]number of genes located in lost regions, both based on UCSC Genome Browser on Human - May 2004 Assembly. [c]Candidate genes were selected on the basis of a database of known cancer genes[26]. [d]miRNA annotation, based on UCSC Genome Browser on Human - May 2004 Assembly and http://microrna.sanger.ac.uk. Losses of 7q11, 8p23-12, 9q34, 15p13-q14, 15q24, 16p13, 20q13 and 22p13-q12 were previously not identified in the study by Joos et al.[14]

Table 3. A comparison of copy number status obtained by array comparative genomic hybridization and FICTION.

Gene	*Chromosomal band*		*Log2-ratio (Array CGH)*[1]	*Copy number analysis by ADM-2*[2]	*Copy number in FICTION*[3]
REL	2p16.1	case 3	0.34	gain	5 (2-7)
		case 5	0.93	amplification	10 (4-20)
CHD1	5q21.1	case 3	0.01	balanced	3 (1-4)
		case 5	0.24	gain	8 (3-12)
CEP6	6p11-6q11	case 3	0.17	gain	4 (1-8)
		case 5	-0.01	balanced	5 (2-17)
TNFAIP3/A20	6q23.3	case 3	-0.23	loss	3 (1-5)
		case 5	-0.30	loss	4 (1-6)
TEK	9p21.2	case 3	0.46	gain	5 (4-8)
		case 5	0.23	gain	6 (3-12)
NOTCH1	9q34.3	case 3	-0.12	loss	2 (1-4)
		case 5	0.16	gain	7.5 (4-14)
CDK4	12q14.1	case 3	0.06	gain	4 (2-7)
		case 5	0.22	gain	7 (1-11)

Copy numbers of selected genes located in regions with genomic imbalances as detected by array CGH were determined by FICTION in two primary cHL cases. CEP6: centromere probe of chromosome 6. [1]Average log2-ratios of the gained or lost chromosomal region containing the gene of interest. [2]Copy number status by statistical analysis (ADM-2) of the chromosomal region where the candidate gene is located. [3]Copy numbers shown as the median (minimum-maximum) of hybridization signals.

Table 4. FISH study of *STAT6*, *NOTCH1* and *JUNB* in primary cHL cases.

Case	Estimated ploidy level	Median copy number	*STAT6* Ratio[a]	Status	Median copy number	*NOTCH1* Ratio[a]	Status	Median copy number	*JUNB* Ratio[a]	Status
13	4	4	1.00	balanced	ND			6	1.50	gain
14	4	4	1.00	balanced	5	1.25	balanced	ND		
15	4	4	1.00	balanced	3	0.75	balanced	ND		
16	4	4	1.00	balanced	ND			ND		
17	3	4.5	1.50	gain	ND			ND		
18	4	5	1.25	balanced	ND			ND		
19	3	3	1.00	balanced	ND			ND		
20	4	4	1.00	balanced	ND			ND		
21	3	4.5	1.50	gain	ND			ND		
22	4	4	1.00	balanced	ND			ND		
23	3	4	1.33	gain	3	1	balanced	ND		
24	4	7	1.75	gain	3	0.75	balanced	ND		
25	4	5	1.25	balanced	NE			ND		
26	3	3	1.00	balanced	4	1.33	gain	ND		
27	3.5	5	1.43	gain	10	2.86	amplification	ND		
28	3	3	1.00	balanced	3	1	balanced	ND		
29	4	6	1.50	gain	3	0.75	balanced	ND		
30	4	NE			4	1	balanced	ND		
31	4	5	1.25	balanced	3	0.75	balanced	ND		
32	2.75	5	1.82	gain	2	0.73	balanced	ND		
33	4	4	1.00	balanced	NE			ND		
34	4	4	1.00	balanced	NE			ND		
35	ND	ND			ND			2		balanced
36	ND	ND			ND			2		balanced
37	ND	ND			ND			4		balanced
38	ND	ND			ND			4		balanced
39	ND	ND			ND			4		balanced
40	ND	ND			ND			6		gain

Ratios between signal numbers of the candidate gene and the estimated ploidy level were obtained for the cases investigated for STAT6 and NOTCH1. Gains were defined by a ratio of >1.3, amplifications by a ratio of >2.0 and deletions by a ratio of <0.7. ND = cases not determined. NE = cases not evaluable. For JUNB, signal copy numbers below 5 were defined as balanced (considering the characteristic tri- to tetraploidy of HRS cells), between 5 and 8 gained, and above 8 amplified. [a]Ratio between median copy number and estimated ploidy level.

chromosome bands (e.g. 6p25 or 6q27), therefore yielding a resolution of approximately 6 Mb at best. According to the criteria used in this study to define imbalances, we could detect and confirm an approximately 156 kb long deletion, thus achieving a much higher resolution than metaphase CGH.

Beyond the obvious higher resolution of array CGH than metaphase CGH, discrepancies between the study by Joos *et al.*[14] and the present report are most likely caused by the problems of using metaphase CGH to study certain chromosomes (e.g. chromosome 19, which was not evaluable by metaphase CGH and was found to be frequently gained in the present study), the different sample sizes and different criteria for case selection.

Several components of constitutively activated signaling pathways in cHL are affected by genomic imbalances

The genomic profiles of primary cHL cases obtained in this study showed that several components of signaling pathways known to be constitutively activated in HRS cells, such as *REL/NFKB, JAK/STAT* and *AP1/JUNB*,[2,33,34] were affected by genomic imbalances, which may influence gene expression by gene dosage effects. Gains of *REL*, *JAK2* and *STAT*[6,19,32,35] were confirmed once more in this study. In addition to *REL*, there were genomic gains of other members of the NFκB family: five of ten cases in the present study displayed gains on chromosome 19q13, where, for example, *BCL3* and *RELB* are located. Two previous studies also indicated that chromosomal gains and breakpoints in the *BCL3/RELB* region are frequent in cHL.[28,36] Four cases showed gains of chromosome 11q13, where *RELA* maps to. One case showed a distinct amplification on chromosome 10q24.32 including *NFKB2* (*Online Supplementary Table S3*). Furthermore, gains of *CARD9* and *TRAF2* on chromosome 9q34 were observed in five of ten cases in this study. *CARD9* binds BCL-10 and activates NFκB.[37] *TRAF2* activates NFkB via *RANK* signaling and is expressed in primary HRS cells.[38] *NOTCH1* maps to the same gained region on chromosome 9q34. Gains of *NOTCH1* were also observed by FISH in another two cases in an independent series of 11 primary cHL cases. Expression of NOTCH1 protein in primary cHL cases is well-known,[39] but genomic amplifications of *NOTCH1* have so far not been described in cHL. Thus, the gain of chromosome 9q34 possibly affects the expression of several genes involved in the pathogenesis of cHL.

JUNB, a proto-oncogene activated by NFκB and constitutively expressed at high levels in primary cHL cases and cHL cell lines,[34] was gained in seven of ten primary cHL cases in the present study. Gains of *JUNB* were additionally found by FISH analysis in two of seven fur-

ther primary cHL cases, thereby suggesting that *JUNB* is not only activated by NFκB, but might also be expressed at elevated levels due to a gene dosage effect.[40]

Constitutive NFκB activity may also be influenced by deletions of chromosome 6q23-24, found in six cases in this study, where *TNFAIP3/A20*, an inhibitor of *NFκB* is located. However, deletions of 6q24 including the *TNFAIP3* gene were recently also reported in about 10% of follicular lymphomas, which is not characterized by strong NFκB activity, and no other somatic mutations were found in this gene in follicular lymphoma.[41] Hence, a mutation analysis of isolated HRS cells is needed to clarify a potential role of *TNFAIP3* in cHL.

SOCS1, an inhibitor of STAT-signaling, frequently mutated in cHL,[42] appeared to be selectively deleted in one case in this study. Two other cases showed a loss of the complete arm 16p, where *SOCS1* is located. A hemizygous deletion and additional mutations in the other allele present a common mechanism inactivating tumor suppressor genes.[43]

Deletions of *CDKN2B* (p15) have so far not been investigated in cHL and homozygous deletions of CDKN2A (p16) appeared only as a subclonal event.[44] However, DNA methylation studies in Hodgkin's lymphoma have shown that *CDKN2B* and *CDKN2A* are frequently targeted by hypermethylation.[45] Thus, deletion and hypermethylation might represent alternative mechanisms leading to *CDKN2B/CDKN2A* inactivation in cHL.

Interestingly, various microRNA are located in regions displaying genomic imbalances in HRS cells (Tables 1 and 2). miR-27a, mapped to chromosome 19p13, which was gained in seven of ten primary cHL cases in the present study, was shown to be one of the most strongly upregulated microRNA in cHL cell lines.[46]

Collectively, the data from the present study show that array CGH of primary HRS cells enables the detection of new genomic imbalances and the delineation of known ones. Furthermore, our findings suggest that genomic aberrations in HRS cells may contribute to the typical cHL-specific phenotype with constitutive expression of several signaling pathways via gene dosage effects.

Authorship and Disclosures

SH: microdissection and acquisition of array CGH data, analysis and interpretation of data, drafting the article; JIMS: FISH/FICTION analysis, analysis and interpretation of data, drafting the article; SG, MG, IN, JR: FISH/FICTION analysis; JH: microdissection, AC, WK, MP, JPM: pathological evaluations, supply of essential material; AB, RK, RS: conception of the study and experimental design, analysis and interpretation of data, critical revision of the article for important intellectual content MLH: pathological evaluations, experimental design. The authors reported no potential conflicts of interest.

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