[CANCER RESEARCH 60, 549–552, February 1, 2000]

Advances in Brief

Genomic Imbalances Including Amplification of the Tyrosine Kinase Gene *JAK2* in CD30$^+$ Hodgkin Cells[1]

Stefan Joos,[2] Manfred Küpper, Sibylle Ohl, Frederike von Bonin, Gunhild Mechtersheimer, Martin Bentz, Peter Marynen, Peter Möller, Michael Pfreundschuh, Lorenz Trümper, and Peter Lichter

Deutsches Krebsforschungszentrum, Abteilung Organisation Komplexer Genome (H0700), D-69120 Heidelberg [S. J., M. K., S. O., P. L.]; Universitätskliniken des Saarlandes, Innere Medizin I, D-66421 Homburg/Saar [F. v. B., M. P., L. T.]; Pathologisches Institut, Universität Heidelberg, D-69120 Heidelberg [G. M.]; Abteilung Innere Medizin III, D-89081 Ulm [M. B.]; Human Genome Laboratory, B-3000 Leuven [P. Ma.]; Abteilung für Pathologie des Universitätsklinikums Ulm, D-89081 Ulm [P. Mö.], Germany

Abstract

Comparative genomic hybridization was applied for a comprehensive screening of frequently occurring net gains and losses of chromosomal subregions in small populations of CD30$^+$ Hodgkin cells and their morphological variants. In 12 Hodgkin's lymphomas, recurrent gains were detected on chromosomal arms 2p, 9p, and 12q (in six, four, and five tumors, respectively) and distinct high-level amplifications were identified on chromosomal bands 4p16, 4q23-q24, and 9p23-p24. In Hodgkin cells with 9p23-p24 amplification, fluorescence *in situ* hybridization revealed an increased copy number of chromosomal sequences spanning the tyrosine kinase gene *JAK2*. Several of the imbalances described, in particular a gain in chromosomal arm 9p that includes *JAK2* amplification, are similar to the genomic changes detected in primary mediastinal B-cell lymphoma.

Introduction

In Hodgkin's disease, four well-defined histotypes of cHL[3] have been distinguished according to the recent REAL classification, *i.e.*, cHL-LR, cHL-NS, cHL-MC, and cHL-LD, in addition to the clinically and immunophenotypically distinct subtype of paragranuloma, or NLPHL (1). All subtypes are characterized by the presence of only a small fraction of malignant cells, referred to hereafter as "Hodgkin cells," which exist in several variants: the mononuclear variant, the L&H cell, and the lacunar cell variant, as well as the multinucleated Reed-Sternberg cell (2). Because of the small numbers, low mitotic index, and frequently poor chromosome morphology of these cells, chromosome banding analysis is particularly difficult and has not revealed specific chromosomal changes that would immediately indicate the localization of genes involved in the etiology of Hodgkin's disease (3). Therefore, we used an alternative approach and collected pools of 30 CD30$^+$ Hodgkin cells from a series of 11 cHLs and a single case of NLPHL. The genomic DNA of the pooled cells was subjected to universal PCR amplification and used as probe for CGH. On the basis of the results obtained, the copy number of a candidate gene, *JAK2*, was analyzed by FISH and Southern blot analysis in Hodgkin cells and PMBL, in which similar chromosomal imbalances have been found previously (4).

Materials and Methods

Tumor Material. Lymph node biopsies from seven male and five female patients were used for cytogenetic analysis of Hodgkin cells. The mean age of the patients at the time of diagnosis was 38.3 years. The 11 cHLs comprised 1 nodular variant of cHL-LR, 4 cases of cHL-MC, 5 cases of cHL-NS, 1 case of cHL-LD, and 1 case of a nodular variant of NLPHL. Tumors were classified according to the guidelines of the pathology panel of the German Hodgkin's study, anticipating the upcoming WHO classification (5) based on routinely stained paraffin sections and immunohistology that included stains for CD3, CD15, CD20, and CD30 as well as epithelial membrane antigen. The tumor staging and immunophenotypes of CD3, CD20, and CD30 are listed in Table 1. From each tumor, fresh, unfixed tissue material was available.

Isolation of Hodgkin Cells. For isolation of Hodgkin cells, lymph nodes derived from Hodgkin's disease were prepared as described previously (6). Briefly, lymphocytes and Hodgkin cells from viable single-cell suspensions were fixed in 3% w/v paraformaldehyde and applied to glass slides by cytospin centrifugation. For immunostaining of CD30 antigen, Alkaline-phosphatase-anti-alkaline-phosphatase assay 4 was applied (6), using monoclonal antibody HRS-4. Identification of Hodgkin cells was based on positive staining for CD30 antigen and on morphological criteria. In the single case of NLPHL, which exhibited large CD20$^+$/CD30$^+$ malignant cells, primarily morphological criteria were applied. Thirty Hodgkin cells were isolated and collected with glass capillaries, using a micromanipulation device (Eppendorf, Hamburg, Germany).

DOP-PCR. Hodgkin cells were first digested with proteinase K (250 μg/ml) in 20 μl of 1× PCR buffer (2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3, 0.1 mg/ml gelatin) for 1 h at 55°C, with subsequent inactivation of the enzyme at 95°C for 15 min. For universal amplification of the genomic DNA, degenerate oligonucleotide-primed DOP-PCR as described by Telenius *et al.* (7) was applied. After the PCR reaction, excessive DOP primers were separated from the amplified genomic DNA on the appropriate columns (Qiagen, Hilden, Germany).

CGH. Preparation of metaphase chromosomes, probe labeling, hybridization, and image acquisition were performed as described previously (4, 8). Chromosomal imbalances were detected based on the ratio profile deviating from the balanced value of 1.0. Chromosomal regions were scored as gains or losses when the ratio profile either reached or exceeded the diagnostic thresholds of 1.25 or 0.75, respectively. Overrepresentations were considered high-level amplifications when the CGH ratio exceeded the value of 2.0 or when the fluorescence showed a strong distinct signal detected by visual inspection and the corresponding ratio profile was diagnostic for overrepresentation. Centromeric regions as well as chromosomes 1p32-p36 and 19 were not scored in the results for reasons specified elsewhere (9). The quality of the CGH experiments was assessed using genomic DNA of male individuals as internal control probes; monosomy of the X chromosome was clearly visible in all experiments performed in this study.

FISH. Hodgkin cells were detected by staining with monoclonal anti-CD30 (HRS-4), a secondary alkaline phosphatase conjugated antibody (Dako Envision; DAKO Diagnostica, Hamburg, Germany) and fast red substrate. Slides were then treated with 1.5% Triton X-100. After denaturation of chromosomal

Received 11/8/99; accepted 12/13/99.

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[1] Supported by the Tumorzentrum Heidelberg/Mannheim (Grants I./I.1. and I./I.2.) and by the Deutsche Forschungsgemeinschaft (Grants Be 1454/5-2, Li 406/4-1, and SFB 399-A8).

[2] To whom requests for reprints should be addressed, at Deutsches Krebsforschungszentrum, H0700, D-69120 Heidelberg, Germany. Phone: 49-6221-424620; Fax: 49-6221-424639; E-mail: s.joos@dkfz-heidelberg.de.

[3] The abbreviations used are: cHL, classical Hodgkin's lymphoma; cHL-LR, lymphocyte-rich cHL; cHL-NS, nodular sclerosis cHL; cHL-MC, mixed-cellularity cHL; cHL-LD, lymphocyte-depletion cHL; NLPHL, nodular lymphocyte predominant Hodgkin's lymphoma; CGH, comparative genomic hybridization; PMBL, primary mediastinal B-cell lymphoma; DOP-PCR, degenerate-oligo-primed-PCR; FISH, fluorescence *in situ* hybridization.

Table 1 *Selected clinicopathological data of investigated cases of Hodgkin's disease*

Patient no.	Sex	Age (years)	Primary/ relapse	Subtype	Stage[a]	Immunophenotype Hodgkin's cells		
						CD3	CD20	CD30
HDK 1	M	53	R	cHL-LR-n	III S	−	−	+
HDK 2	M	33	P	cHL-MC	III A	−	−	+
HDK 3	M	49	P	cHL-MC	I A	−	−	+
HDK 4	F	21	R	cHL-MC	III	−	−	+
HDK 5	M	63	P	cHL-MC	II B	−	NA[b]	+
HDK 6	F	32	P	cHL-NS	II A	−	−	+
HDK 7	M	26	P	cHL-NS	NA	−	−	+
HDK 8	F	16	P	cHL-NS	IV A	−	−	+
HDK 9	F	37	P	cHL-NS	III B	−	NA	+
HDK 10	M	14	P	cHL-NS	II A	−	−	+
HDK 11	F	78	P	cHL-LD	I B	−	−	+
HDK 12	M	38	P	NLPHL-n	NA	−	+	+

[a] Staging was performed according to the Ann-Arbor system.
[b] NA, not analyzed.

DNA in 70% formamide at 76°C for 5 min, specimens were dehydrated in a series of ethanol solutions and air dried. As probes, PAC clones PJ2A and PJ2B were used, spanning the entire *JAK2* gene as well as ~50 kb of the downstream region on chromosome 9p24 (10). Probe labeling and *in situ* hybridization were performed as described previously (4).

Southern Blot Analysis. Genomic DNA was isolated from peripheral blood, PMBL-derived cell line MedB-1, and a PMBL tumor harboring a distinct high-level amplification of chromosomal band 9p23-p24 (PMBL 16; Fig. 2; Ref. 4). DNA preparation and Southern blot analysis were performed as described elsewhere (11), using a 880-bp *Hin*dIII fragment probe of *JAK2* c-DNA. As an internal control, a 2.1-kb *Eco*RI cDNA clone of *WNT2* (American Type Culture Collection, Rockville, MD) located on chromosome 7q31-q32 was used. The level of amplification was calculated by comparison of the hybridization signal intensities of test and control signals on X-ray films using the software application TINA 2.0 (Raytest, Straubenhardt, Germany).

Results

CGH Analysis. Universally amplified genomic DNA of 30 $CD30^+$ Hodgkin cells from 12 different Hodgkin's lymphomas was analyzed by CGH. The net chromosomal gains and losses of chromosomal regions that where identified are summarized in Fig. 1. Fifty-three imbalances were detected in Hodgkin cell pools in 11 of 12 of the analyzed tumors, with an average number of 4.4 imbalances per case (range, 0–8 imbalances). Gains were detected much more frequently than losses: 48 gains (4.0 per tumor) *versus* 5 losses (0.4 per tumor). Chromosomal losses included 17p (2 of 12 tumors), 4q32-qter, 13q31-qter, and 18p (1 of 12 tumors). Overrepresentations most frequently involved chromosomal arms 2p in 6 of 12 tumors, 12q in 5 of 12 tumors, and 9p in 4 of 12 tumors. Twenty-two chromosomal arms were involved in low-frequency gains. High-level amplifications of distinct chromosomal subregions were detected in three different tumors on chromosomal bands 4p16, 4q23-q24, and 9p23-p24 (Fig. 2, *a–c*). Both amplifications on chromosome 4 affected cHLs (HDK 4 and HDK 7), whereas the one on chromosome 9p was found in the NLPHL subtype (HDK 12).

Detection of *JAK2* Copy Number Amplification in NLPHL and PMBL. The amplified chromosomal region 9p23-p24 constitutes the consensus region that is recurrently gained in neoplastic cells of Hodgkin's lymphomas in ~33% and PMBL in ~50% of cases (4, 12). One candidate gene at this site, *JAK2*, codes for a non-receptor tyrosine kinase that plays a key role in cytokine signal transduction. To determine whether this gene is involved in the high-level amplification of NLPHL, case 12 (Fig. 2*c*), FISH with two PAC clones covering the *JAK2* genomic region was applied (Fig. 3). In 24 $CD30^+$ Hodgkin cells evaluated, an average signal number of 18.5 ± 10.3 was detected. In addition, hybridization signals were frequently clustered in a domain-like signal pattern. Both the high signal number and the spatial signal distribution strongly indicate amplification of the genomic region of *JAK2* in these cells. Cells surrounding the $CD30^+$ cells exclusively exhibited two *JAK2* signals, *i.e.*, were in disomic state.

Whereas Southern blot analysis is prohibited by the small fraction of Hodgkin cells in primary Hodgkin's lymphomas, it is a feasible approach for PMBL. The copy number of *JAK2* was assessed in genomic DNA of peripheral blood cells, cells from a PMBL-derived cell line (MedB-1), and cells from a primary PMBL tumor (PMBL 16) harboring a high-level amplification of 9p23-p24 (Fig. 2*d*; Ref. 4). Using a *JAK2* cDNA fragment as probe, six hybridization signals were detected in *Hin*dIII-digested genomic DNA (3, 3.3, 4.5, 5, 6.6, and 9.3 kb in length). Measurement of the hybridization signals of *JAK2* and signals of an internal control probe *WNT2* (~4.5, 6, 7.5, and 8 kb in length) revealed an ~19-fold higher intensity in PMBL 16 and a 4-fold higher intensity in MedB-1 compared with normal DNA (Fig. 4).

Discussion

In the present study, chromosomal imbalances were detected in small populations of Hodgkin cells by a combined approach of universal PCR and CGH. The feasibility of this method has been shown previously, (*e.g.*, Ref. 13). The protocol applied here was thoroughly assessed prior to the present study, demonstrating that we could reliably obtain valid CGH data from ≥20 pooled cells (data not shown).

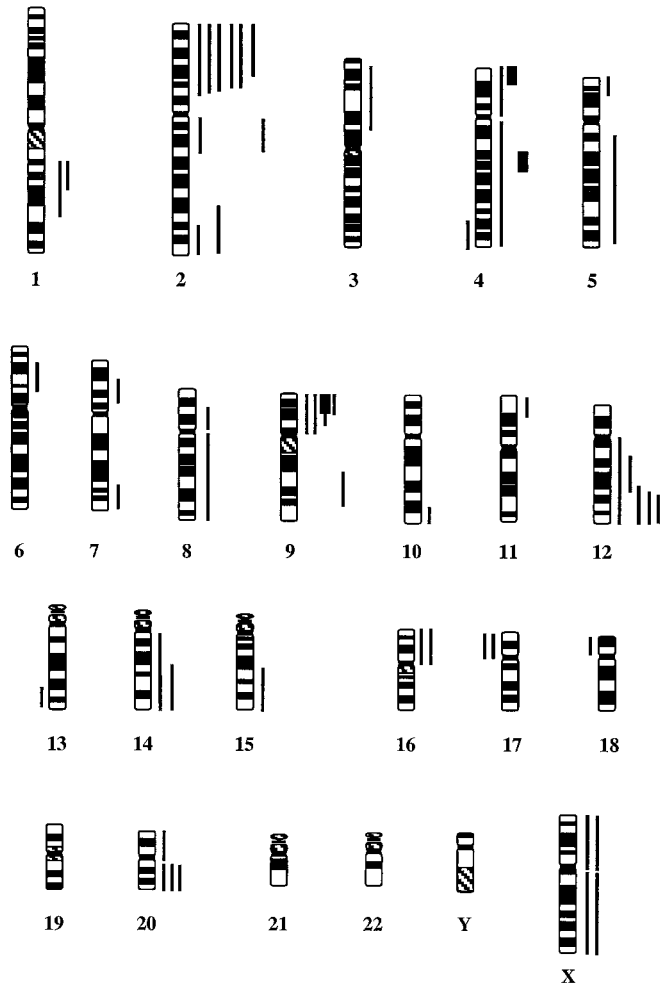

Fig. 1. Summary of the CGH data of Hodgkin cells isolated from 12 cases of Hodgkin's disease. Chromosomal gains are indicated by *vertical lines* to the *right*, losses by *vertical lines* to the *left* of the chromosome ideograms. Distinct high-level amplifications are indicated as *bold bars*.

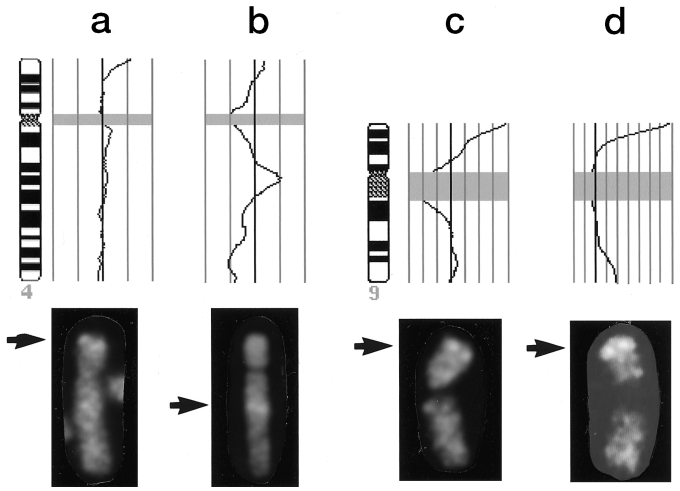

Fig. 2. Examples of CGH experiments showing the detection of distinct amplified genomic sequences in Hodgkin cells (*a–c*) and PMBL cells (*d*). *Top*, average ratio profiles; *bottom*, examples of hybridized chromosome homologues. *Arrows* indicate amplification of subbands 4p16 in HDK 7 (*a*), 4q23-q24 in HDK 4 (*b*), 9p23-p24 in HDK 12 (*c*), and 9p23-p24 in PMBL 16 (*d*; Ref. 4). Centromeric regions shaded in *gray* are excluded from evaluation for reasons indicated elsewhere (9). *Vertical lines* indicate average ratio value of 1 (*black vertical line*) and diagnostic cutoff values for gains (*e.g.*, 1.25, 1.5; *gray vertical lines* on *right*) and losses (*e.g.*, 0.75, 0.5; *gray vertical lines* on *left*).

Despite of the restricted number of Hodgkin's lymphomas investigated, a distinct pattern of chromosomal imbalances emerged from this analysis. Gains were detected on chromosomal arms 2p, 9p, and 12q in 33–50% of cases, whereas most other chromosomes were affected in only one or two tumors. CGH also identified amplified DNA sequences from distinct chromosomal subregions, *i.e.*, on 4p16, 4q23-q24, and 9p23-p24. The characteristic chromosomal imbalances as well as the distinct high-level amplifications previously were not known to be affected in Hodgkin cells.

A very recent CGH analysis on flow-sorted $CD30^+$ Hodgkin cells describes a higher number of imbalances than were found in the present study (14). However, many of those imbalances seem not to be characteristic because most chromosomal regions were over- and underrepresented at similar frequencies. In addition, the most frequent aberrations affect chromosome regions known to be difficult in CGH analysis (*e.g.*, pericentromeric regions of chromosomes 1 and 9) and therefore are not considered in CGH evaluation (9).

The unbalanced chromosomal regions detected in the present study include several interesting candidate genes, for example: (*a*) receptor tyrosine kinase *ALK* (2p23), which was found to be involved in t(2;5) translocations of ~40% of $CD30^+$ anaplastic large B-cell lymphomas (15); (*b*) the members of the REL/NF-κB family of transcription factors, *REL* (2p15) and *NF-κB*-p50 (4q23-q24), which have been directly implicated in the pathogenesis of Hodgkin's lymphoma (16); (*c*) fibroblast growth factor receptor 3 (*FGFR3*) and multiple myeloma SET domain gene *MMSET* (4p16), which were found in breakpoint regions of t(4;14)(p16;q32) translocations in multiple myeloma (17); and (*d*) *MDM2* (12q14), which is a potent inhibitor of *TP53* and is involved in cell cycle control.

It is intriguing that the prominent finding of this study, a gain in chromosome arm 9p in one-third of the cases, is in concordance with previous data of PMBL that revealed a gain of 9p in half of the cases (4, 12). This genomic change is rare in other B-cell non-Hodgkin's lymphomas and was observed only in 7 of >300 cases of B-cell non-Hodgkin's lymphomas other than PMBL (18). The high-level amplifications in one Hodgkin case and one PMBL allowed us to narrow the consensus region on 9p to subbands 9p23-p24. Two candidate genes are located within this area. The first, *NF1B*, encodes for a transcription factor and is involved in chromosomal translocations in pleomorphic adenomas of the salivary gland (19). The second, *JAK2*, is involved in the Jak/STAT signal transduction of various cytokines as well as Ras-dependent pathways (20). We could demonstrate that *JAK2* is in fact coamplified in each of the tumors with 9p23-p24 amplification. In addition, the frequent gains of chromosome arms 2p and 12q in Hodgkin cells were found recurrently in ~30% of PMBL tumors as well (4). On the basis of these similarities,

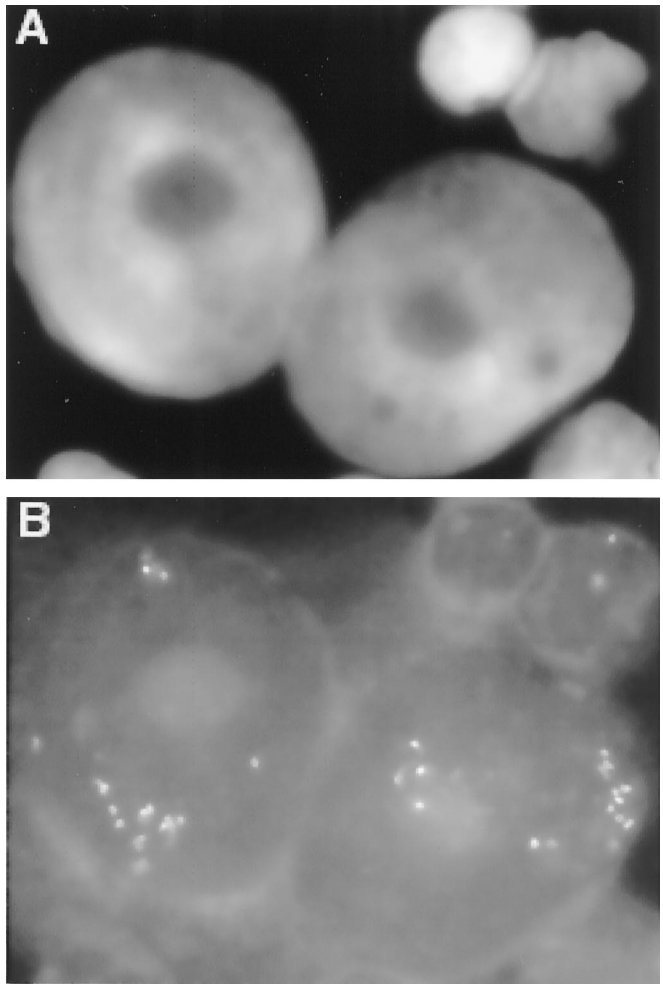

Fig. 3. Interphase FISH of Hodgkin cells and adjacent cells in NLPHL case HDK 12. As probes, two PAC clone representative for the *JAK2* genomic region on chromosome 9p23-p24 were used. Neoplastic cells were identified by positive staining with anti-CD30 antibody (not shown). *A*, images of Hodgkin cells and smaller adjacent cells visualized with 4′,6-diamidino-2-phenylindole; *B*, detection of multiple *JAK2* signals in large Hodgkin cells, indicating that *JAK2* is included in the amplicon on 9p23-p24.

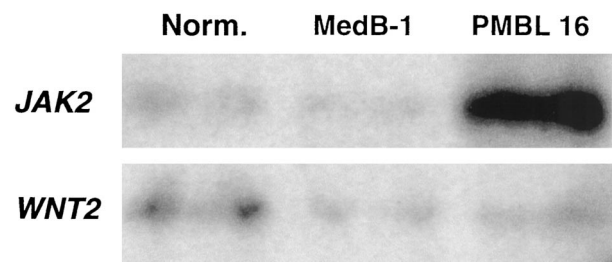

Fig. 4. Southern blot analysis of *Hin*dIII-digested normal genomic DNA of peripheral blood (*Norm.*), DNA from PMBL-derived cell line MedB-1, and DNA from PMBL 16, using a *JAK2* cDNA probe as well as the internal control probe *WNT2*. *JAK2* is amplified ~4-fold in PMBL cell line BHD-1 and 19-fold in the primary tumor PMBL 16. Only the 6.6-kb signal of *JAK2* and the 4.5-kb signal of *WNT2* are shown.

it is tempting to speculate that Hodgkin's disease and PMBL might share common pathogenetic pathways. In line with this hypothesis is the finding of rare cases of composite lymphomas with features of Hodgkin's disease and PMBL (21). In addition, both tumor entities share a number of clinical and immunological features, *e.g.*, their frequent mediastinal origin and the lack of functional expression of HLA class I and immunoglobulin molecules.

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Cancer Res 2000;60:549-552.