Common features and differences in the transcriptome of large cell anaplastic lymphoma and classical Hodgkin's lymphoma

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Background and Objectives. Anaplastic large cell lymphoma (ALCL) and classical Hodgkin's lymphoma (HL) are derived from different cell types, namely T cells and B cells, respectively. However, both lymphomas share a similar cytological and immunohistochemical tumor cell phenotype with little resemblance to their cells of origin.

Design and Methods. In this study, the transcriptional profiles of ALCL cell lines, primary ALCL tumor cells from peripheral blood and HL cell lines were compared to each other and to normal B-cell subsets, B non-Hodgkin's lymphomas (NHL) and B NHL- and Epstein-Barr virus (EBV)-transformed B-cell lines in order to establish their relationship at the transcriptional level and to identify genes with possible pathobiological impact. Expression of some of the genes identified was confirmed in microdissected primary tumor cells by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry.

Results. HL samples clustered separately from ALCL samples, but HL and ALCL were found to be more closely related to each other than to any normal or malignant B-cell sample in the dataset. Their relationship was determined to a large extent, but not exclusively, by lack of expression of B-cell antigens and by the over-expression of mRNA encoding activation markers and structural proteins. Apart from established differences between HL and ALCL, further genes of interest could be identified that distinguish both entities from each other and from the other samples. The differential expression of PRAME, DDR2, SOCS3 and CEBPD in HL and ALCL was confirmed in primary tumor tissue by immunohistochemistry and/or RT-PCR.

Interpretations and Conclusions. At a transcriptional level HL is more closely related to Alk$^+$ ALCL than to the B-NHL or B-cell samples investigated, although it is a B-cell derived lymphoma. The newly identified genes discriminating HL and ALCL may be pathobiologically important and may serve as possible therapeutic targets.

Key words: Hodgkin's lymphoma, non-Hodgkin's lymphoma, microarray.

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Anaplastic large cell lymphoma (ALCL) and classical Hodgkin's lymphoma (HL) are pathologically distinct entities. However, there is considerable overlap in the pathophisiological features of these diseases.[1] The tumor cells of both entities share cytological features with each other, as well as the expression of CD30.[2] ALCL with large tumor cells, partial lymph node involvement and fibrosis may resemble nodular sclerosis HL. *Vice versa*, tumor cells may be numerous in HL, resembling the infiltrate of ALCL. ALCL is a neoplasm derived from T cells in most if not all cases.[3] Morphologically, the lymphoid tumor cells of ALCL have large pleomorphic nuclei and abundant cytoplasm. A broad morphologic spectrum of ALCL exists, including a small cell, lymphohistiocytic and *common* variant.[4] The tumor cells of ALCL grow in sheets and disrupt the normal lymph node architecture. Extranodal sites are frequently involved in ALCL. The majority of cases of ALCL display aberrant expression of anaplastic lymphoma kinase (ALK). ALK is a receptor tyrosine kinase of the insulin receptor superfamily and is normally absent in lymphoid cells. The aberrant expression of ALK in ALCL is caused by chromosomal translocations involving the ALK gene-locus on chromosome 2 and various fusion partners, the most common of which is the nucleophosmin gene on chromosome 5.[4] Dimerization of the chimeric proteins formed leads to autophosphorylation of the catalytic domain of ALK and its oncogenic activity.[5] Some ALCL cases, however, lack expression of ALK despite similar morphology.[4] HL is morphologically characterized by a minority of large mononuclear Hodgkin- and multinucleated Reed-Sternberg (HRS) cells surrounded by a heterogeneous cellular infiltrate.[6] It has been shown that the HRS cells represent the clonal tumor cell population.[7] In all cases of lymphocyte-predominant HL and in the vast majority of classical HL the tumor cells are derived from B cells.[7] Only in rare cases of classical HL do HRS cells represent transformed T cells.[8-10] Despite their lymphoid origin, HRS cells display a mixed phenotype with the expression of myeloid, dendritic cell and T- or NK cell markers.[11-13] Common B-cell markers have usually been lost in HRS cells.[7,14] Unlike in ALCL or other lymphomas, no characteristic chromosomal aberration has been detected as a common transforming event in HL. Considering the different cellular origin and different genetic characteristics of HL and ALCL, the diagnostic overlap in these entities does not represent a true biological gray area. However, as both ALCL and HL show hardly any resemblance to their cells of origin, but present with a similar cytological phenotype, a comparison of the two entities promises to be interesting. So far, such a comparison has been performed on clinical, morphological, immunohistochemical

and molecular levels in many studies.[15] With the advent of broad scale molecular profiling techniques in recent years novel genes distinguishing HL and ALCL were detected with microarrays containing probes for up to 1176 mRNA.[16-19] In the present study we compared the transcriptome of both entities, using a large-scale microarray-based approach. Since tumor cells in primary tissue of HL are a minority surrounded by a polymorphous cellular infiltrate it is not feasible to obtain large amounts of RNA from purified primary HL tumor cells. Therefore, gene expression profiles (approximately 9000 genes) were generated from classical HL and ALCL cell lines and a sample of primary leukemic ALCL tumor cells and compared to each other and to profiles of the main subsets of normal mature B cells and various B-cell non Hodgkin's lymphomas (NHL); the profiles of the latter were available from the literature.[20] The expression of several markers identified in the analysis was investigated in primary tissue of ALCL and HL by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. The following questions were addressed in the study: given the different origin but morphologic similarity of ALCL and HL, do the gene expression profiles identify these two lymphomas as related disorders? Can genes regulated in common in ALCL and HL be identified and do these distinguish ALCL and HL from B NHL? How do the gene expression profiles reflect the different biology of ALCL and HL? With the two latter questions, we sought to detect genes that might be relevant for the pathogenesis of these tumors.

Design and Methods

Cell lines and culture conditions

The ALCL cell lines used were established from pleural effusions (SR786, Su-DHL1) or peripheral blood (Karpas299) of patients with ALCL-type T cell lymphoma carrying the characteristic t(2;5) translocation described above.[21] The ALCL cell lines were obtained from the Deutsche Sammlung für Mikroorganismen und Zell-Linien, Braunschweig, Germany. The HL cell lines originate from patients with HL of nodular sclerosis (L428 and HDLM2) or mixed cellularity subtype (KMH2 and L1236).[22-24] HDLM2 is of T-cell origin, whereas the three other HL cell lines are of B-cell origin, as shown by Southern blot and/or PCR analysis of antigen-receptor gene rearrangements.[22-24] Cell lines were grown in RPMI 1640 with Glutamax-1 (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (Karpas299, Su-DHL1, L428, L1236, KMH2), 15% fetal calf serum (SR786) or 20% fetal calf serum (HDLM2) and 100 U/mL penicillin/streptomycin at 37°C in an atmosphere containing 5% CO_2.

Primary ALCL tumor cells

The diagnosis of ALCL was established on a lymph node biopsy from a 30-year old female patient. The architecture of the lymph node was effaced and the node contained an infiltrate of medium to large lymphoid tumor cells. By conventional immunohistochemical analysis using a streptavidin-biotin detection system with alkaline phosphatase and fast red as chromophore the tumor cells were found to be $CD30^+$, $ALK1^+$, $CD3^-$, $CD15^-$, $CD20^-$, $Ig\kappa^-$, $Ig\lambda^-$ (all antibodies from DakoCytomation, Glostrup Denmark, except CD3: Novocastra, Newcastle upon Tyne, UK; and CD15: Quartett, Berlin, Germany). The patient suffered a leukemic form of ALCL so primary tumor cells could be obtained from peripheral blood. Mononuclear cells isolated by Ficoll-Hypaque density gradient were shown to be 95% tumor cells by flow cytometry (CD2: 99%, CD3: 94%, CD4: 98%, CD8: 1%, CD11c: 4%, CD19:0.5%, CD57: 3%, Ki67: 96%, TCRα/β 2.5%, TCR/γ/δ: 0%; FACScan, Becton Dickinson, Heidelberg, Germany; all antibodies from Becton Dickinson, except for Ki67: DakoCytomation).

B-cell subsets

The isolation of the primary B cells used in this analysis has been described in detail elsewhere.[25] Briefly, normal B-cell subsets were isolated by magnetic cell separation using a MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the expression of the following markers: naïve B cells ($CD27^-$, $CD10^-$, $CD3^-$, $CD38^{low}$, $CD14^-$, IgD^+), centroblasts ($CD77^+$, $CD38^{high}$), centrocytes ($CD77^-$, $CD38^{high}$, $CD39^-$, $CD3^-$, $CD10^+$), and memory B cells ($CD10^-$, $CD3^-$, $CD38^{low}$, $CD14^-$, $CD27^+$). Tumor cells of primary B-NHL had been enriched by magnetically depleting T cells, NK cells, monocytes and other non-tumor cells as described in detail elsewhere.[25]

Generation of cRNA and microarray hybridization

Microarray hybridization was performed as recommended by the manufacturer, starting from 5 μg of purified total RNA and hybridizing each 15-μg biotin-labeled cRNA to U95A microarrays (Affymetrix, High Wycombe, UK). After scanning, the expression values for the genes were determined using Affymetrix Microarray Suite 5.0 software, applying its statistical algorithm and its global scaling option. Microarray data from B-NHL, B-cell subsets and HL have been reported previously.[14,20,26,27]

The primary data are available in the GEO-database under the accession numbers GSE4109, GSM93960, GSM93963, GSM93965, GSM93966 and on *http://icg.cpmc.columbia.edu/Web_Data/JCI/2003_JCI.htm*

Biostatistical analysis

For the comparison of ALCL and HL profiles with B-cell subsets and B-NHL the data were loaded into the GeneSpring software (Silicon Genetics, Redwood City, CA, USA) as recommended, applying per chip normalization using the distribution of all genes and per gene normalization using the median of each gene. The hierarchical clustering algorithm used to generate the dendrogram is based on the average linkage method. Only genes showing at least four samples with a present score and a minimum fold change of five compared to the other samples were considered for construction of the dendrogram in order to limit the data to genes with a high difference in expression. To identify which genes are expressed similarly in HL and ALCL in comparison to the other samples a supervised analysis was performed applying a one way analysis of variance (ANOVA) test with Bonferroni's correction of multiple testing.[28] For this analysis, HL and

ALCL samples were defined as one group, and all the other samples as the second group. The cut-off score was set to a minimum fold change of two between these groups in order to detect genes of biological relevance. The direct comparison of ALCL and HL was limited to four samples in each group. A high degree of variance in the expression of single genes was observed within each of these groups. Applying a statistical test with such a limited number of samples for the two groups seemed inappropriate. Therefore, sixteen pairwise comparisons were performed with each of the four ALCL samples being compared to each of the four HL samples. Matching cells on two arrays were directly compared. The algorithm computes a *p*-value, applying a signed rank test on the perfect match and mismatch differences for each of 15 probe pairs in a probe set (a probe pair consists of an oligonucleotide perfectly matching an interrogated mRNA and a corresponding oligonucleotide carrying one mismatch in the middle; on the U95 array a probe set consists of 15 different probe pairs for each mRNA). The resulting *p*-values are used to determine the difference in gene expression. A minimum fold change of two in each of the comparisons was considered to be biologically relevant.

RT-PCR analysis of selected genes in microdissected primary lymphoma cells

Frozen tissue of cases diagnosed with ALCL, HL or benign lymphadenitis or tonsillitis were selected from the files of the Department of Pathology of the University of Frankfurt. The diagnoses of ALCL and HL had been made according to the WHO lymphoma classification. All tissues used in this study were taken for diagnostic purposes. The study was approved by the ethical committee of the University Clinic Frankfurt.

Micromanipulation and cDNA synthesis were carried out as described before.[20] Briefly, 5 μm thick sections were mounted on membrane-covered slides and stained using a rapid hematoxylin and eosin protocol. For HL cases 50 single HRS cells and for ALCL and non-neoplastic control tissue, sheets of 50 cells were dissected and transferred into Purescript cell lysis buffer (Biozym, Hamburg, Germany) using a UV laser beam (PALM, Bernried, Germany). Non-neoplastic control tissue consisted of lymphoid tissue from tonsil or lymph nodes containing a mixture of B cells and T cells from both lymphoid follicles with germinal centers and interfollicular areas. Total RNA was isolated using the Purescript RNA Isolation Kit (Biozym) with the modifications described. cDNA was generated with random hexamer priming using the first strand cDNA synthesis kit for RT-PCR (AMV, Roche Applied Science, Indianapolis, IN, USA). Semi-quantitative RT-PCR analysis for PRAME was carried out using a semi-nested approach as described before.[20] Briefly, a first round of 20 cycles of PCR was carried out with the total cDNA obtained from 50 microdissected cells. One microliter of the first round reaction mixture was subjected to a second round of 40 cycles of PCR with an internal reverse primer. PCR-products were analyzed by agarose gel-electrophoresis. Quantitative RT-PCR was carried out for the CEBPD/NF-IL6β, DDR2, SOCS3, SEC7, SMARCA-2 and TC21/RRAS2 genes using commercially available FAM-labeled assays (Assay on demand, Applied Biosystems, Foster City, CA, USA). 18s rRNA, used as a housekeeping reference gene, was co-amplified with VIC-labeled probes in the same reaction. The total cDNA obtained from 50 microdissected cells was subjected to the PCR reaction. For each gene and each case three samples containing 50 cells were analyzed separately. Amplification of expressed genes was detected by real-time PCR (7500 Real-Time PCR system, Applied Biosystems).

Immunohistochemical staining of primary HL and ALCL cases

Paraffin-embedded lymph node tissues with infiltration of HL and ALCL were selected from the files of the Institute of Pathology, Frankfurt. All tissues had been submitted for diagnostic reasons. The diagnoses of HL and ALCL had been established by routine hematoxylin/eosin- and Giemsa staining and immunohistochemical staining with antibodies for CD3, CD15, CD20, CD30 and Alk1. All tissues were studied in accordance with national ethical principles. Tissue sections 2 μm thick were cut, mounted on glass slides (Superfrost Plus, Microm, Walldorf, Germany) and dewaxed through a graded alcohol series. Slides were blocked with normal goat serum (4 μg/mL; Santa Cruz Biotechnology, Heidelberg, Germany) for 15 min and incubated overnight at room temperature with primary rabbit polyclonal antibodies for the detection of DDR2 (as described previously:[29] H108, diluted 1:50, Santa Cruz and GEA4023, diluted 1:250, Genex Bioscience, Hayward, CA. USA), CEBPD (M-17, diluted 1:100, Santa Cruz) or SOCS3 (diluted 1:50, kind gift of DJ Hilton, Victoria, Australia). For antigen retrieval slides were cooked in a microwave oven in 600 mL of 10 mM Na citrate, pH 6.0, for 15 min (GEA4023, M-17, SOCS3) or 30 min (H108). Slides were developed using the Envision system (DakoCytomation), with either alkaline phosphatase and Fast Red or horseradish peroxidase and 3.3-diaminobenzidine (Dako).

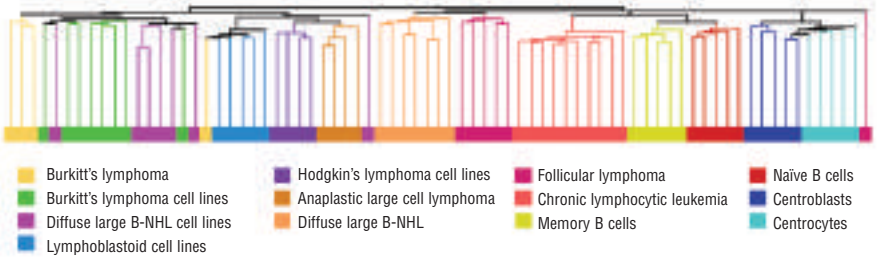

Figure 1. HL is more closely related to ALCL than to the B-cell derived samples in the dataset. The gene expression profiles of HL-, ALCL- and other NHL-cell lines and various NHL, normal B-cell subsets and lymphoblastoid cell lines were hierarchically clustered in an unsupervised analysis. The dendrogram was constructed using 2197 probes. Different sample types are color-coded by diagnosis.

Figure 2. Expression of activation markers and structural proteins and lack of expression of B-cell genes are common to HL and ALCL. The mRNA that were detected in a supervised analysis performed to identify genes differentially expressed in HL and ALCL as compared to all other samples are shown in rows. Genes with a higher expression include those that are also upregulated in lymphoblastoid cell lines (LCL). Arrows indicate ten genes with significantly higher expression when looking at HL and ALCL only. Color changes within a row indicate expression relative to the average of all samples (see methods for algorithm and criteria; red: high expression, green: low expression). From left to right ALCL samples and HL cell lines are: Karpas299, primary ALCL sample, SR786, Su-DHL1, HDLM2, KMH2, L1236, L428.

Results

At a transcriptional level HL was more closely related to ALCL than to the B-NHL and B-cell samples

Gene expression profiles of HL and ALCL cell lines and an ALCL tumor sample were obtained using an oligonucleotide microarray containing probes for more than 12000 mRNA relating to approximately 9000 different genes. These microarray data were compared to gene expression profiles of several subtypes of B NHL (Burkitt's lymphoma, chronic lymphocytic leukemia, diffuse large cell lymphoma, follicular lymphoma, mature B-cell subsets (naïve and memory B cells, centroblasts and centrocytes) and cell lines derived from Burkitt's lymphoma and diffuse large cell lymphoma, as well as EBV-transformed peripheral blood B-cell lines (LCL). Microarray data from B NHL, B-cell subsets and HL have been reported previously.[14,20,26,27]

In an unsupervised hierarchical clustering of all samples, using 2197 genes with a high difference in expression (for criteria see material and methods), the main branches comprised the normal B-cell subsets and most primary tumor probes on one branch and the cell lines, primary Burkitt's lymphoma and the primary ALCL sample from peripheral blood on the other branch (Figure 1). This separation of cell lines and primary tumor samples may be attributed to the activated phenotype of the former. Since the primary Burkitt's lymphoma samples and the ALCL sample cluster together with the cell lines, this separation is not, however, caused only by the somewhat artificial nature of cell lines. All ALCL samples, one primary ALCL and the three cell lines, were found together in a branch. Thus, the transcriptional profiles identify these samples as a group separate from the others. Similarly, all Hodgkin cell lines group together and this branch is found next to

that of the ALCL samples, implying that the gene expression pattern of HL resembles that of ALCL-type T-cell lymphoma more than that of any B-cell sample. However, the T-cell-derived HL cell line HDLM2 groups together with the B cell-derived HL and not with the T-cell-derived ALCL. The LCL samples were found next to HL and ALCL.

Genes distinguishing ALCL and HL from B cells and B-NHL

A supervised analysis was performed in order to identify genes that show a similar expression pattern in HL and ALCL and that distinguish them from the other samples. Forty-two mRNA relating to 32 different genes were detected that are not expressed in HL and ALCL but in the other samples. Most of the genes are typical for B cells or common lymphoid or hematopoietic genes (Figure 2). In order to test whether the relationship of HL and ALCL is only determined by the lack of expression of B-cell and hematopoietic markers, we excluded genes known to be expressed in B cells from the dataset and performed a new unsupervised cluster analysis. Again, HL and ALCL were more closely related to each other than to the other samples (*not shown*; for HL, ALCL and LCL samples the dendrogram is identical to Figure 1). Only ten genes were identified that display a higher expression in HL and ALCL and distinguish them from the other samples. Many of the genes that are expressed at higher level in HL and ALCL are also expressed in the LCL that cluster next to them (Figure 2). These are genes associated with an activated cell type, such as CD30 and ATF3, and many genes involved in morphogenesis and constituents of the cytoskeleton, namely fascin, dystonin, myoferlin, radixin and catenin. Furthermore, genes with possible regulatory functions, such as the transcription factor IER3/IEX1, the suppressor of cytokine signaling (SOCS) 2 and receptors for IL1, IL2 and IL15, were detected.

Identification of genes differentially regulated in ALCL and HL

In order to identify genes that distinguish HL and ALCL, gene expression profiles of four HL-derived cell lines were each subjected to a pairwise comparison with each of four profiles obtained from three ALCL cell lines and one primary tumor sample. Genes whose expression differed by more than 2-fold in all 16 different comparisons were considered for further analysis (see material and methods for detail). Forty-five distinct genes were found to be expressed at a higher level in HL and 34 different genes displayed a significantly higher expression in the ALCL samples (Table 1). Several genes known to be differentially expressed in HL and ALCL were detected, confirming the applicability of the approach. HL cell lines displayed a higher expression of the receptors CD40, CD54 and CD80 and the cytokines lymphotoxin-α (LTA) and interleukin 6 (IL6) and the chemokine CCL17 (TARC), all of which have been reported previously.[30-34] In the ALCL samples, expression of ALK and CD45 was observed, both known to be found in ALCL.[35,36] In addition, the cytotoxic T-cell markers perforin and granzyme B were expressed at a higher level in the ALCL samples than in HL, in line with previous reports.[37,38] The other transcripts with higher expression in HL include possibly interesting genes with regulatory function, such as the transcription factors H-PLK, ZNF354A, FOXO3A, NRF3, SMARCA-2, the receptor tyrosine kinase DDR2, the imprinted gene IPL/TSSC3, the tumor antigen PRAME and genes involved in signaling, such as TC21/RRAS2, SMG-1 and DPYSL2. Of these, IPL, NRF3 and PRAME have been previously detected as HL-specific in the same dataset of HL cell lines in a comparison with B-cell populations.[20] The genes identified as being more highly expressed in ALCL included the transcription factors CEBPD/NF-IL6β, ZFP36, FOSL2 and SEC7, the caspase-1, genes involved in signaling, such as IL1RAP, LTBP1, RAB32 and SOCS3, and genes involved in cell cycle regulation such as cyclin-A1 and GOS2.

Analysis of HL and ALCL samples by RT-PCR

The expression of the genes DDR2, TC21/RRAS2, PRAME, SMARCA-2, CEBPD/NF-IL6β, SEC7 and SOCS3 was also analyzed by RT-PCR. For six of these seven genes, the gene expression results of the microarrays could be confirmed in the cell lines by RT-PCR. Only for SMARCA-2 no apparent difference in gene expression, as detected on the microarray, could be identified by RT-PCR (Figure 3). The gene expression of the other six genes was further investigated in primary tumor tissue. Tumor cells were microdissected from frozen sections of primary tumor tissue with a laser beam. RNA was extracted from these cells and subjected to a semiquantitative RT-PCR (PRAME) or a quantitative RT-PCR (all others). Three cases of HL investigated for the expression of PRAME were positive, whereas all of three primary ALCL cases were negative (*data not shown*). A difference in gene expression in primary HL and ALCL could also be confirmed for the DDR2, CEBPD/NF-IL6β and SOCS3 genes (Figure 3). Although differentially expressed in the cell lines, the genes TC21/RRAS2 and SEC7 displayed equal levels of expression in primary tissue of both tumors (ALCL and HL) and normal lymphoid tissue by RT-PCR (Figure 3).

Immunohistochemical analysis of primary HL- and ALCL tumor tissue

The expression of DDR2, CEBPD and SOCS3 was analyzed at the protein level by immunohistochemical staining of lymph node tissue infiltrated by HL and ALCL. The results of these stains are given in Table 2. The differential expression of these genes in HL and Alk$^+$ ALCL was confirmed (Figure 4). However, SOCS3 was also expressed in a fraction of cases of HL and a few cases of ALCL express the receptor tyrosine kinase DDR2 in addition to Alk (Table 2). Immunohistochemical staining of HL cases for DDR2 and SOCS3 had been performed in previous studies.[29,39]

Discussion

In this study, the transcriptional profiles of ALCL cell lines, primary ALCL tumor cells from peripheral blood and HL cell lines were compared to each other and to normal B-cell subsets, B-NHL and B-NHL cell lines and LCL. The HL cell lines were found to be more closely related to ALCL than to the normal or malignant B-cell samples.

Table 1. Genes that are differentially expressed in HL and ALCL.

Hodgkin's lymphoma cell lines			ALCL		
Probe Set ID	*Fold Change*	*Gene Symbol*	*Probe Set ID*	*Fold Change*	*Gene Symbol*
1183_at	110.71	CCL17/TARC	37137_at	474.11	GZMB/granzymeB
38299_at	95.72	IL6	38326_at	202.46	GOS2
36650_at	52.82	CCND2	38543_at	171.22	ALK
39696_at	44.95	PEG10	1814_at	93.93	TGFBR2
32827_at	41.09	TC21/RRAS2	37391_at	64.40	CTSL/cathepsinL
157_at	33.71	PRAME	1052_s_at	41.92	CEBPD
41294_at	32.64	KRT7	41354_at	36.62	STC1
35004_at	29.09	DHRS2	31792_at	33.29	ANXA3/annexin3
35839_at	26.35	SQLE	40742_at	31.33	HCK
36296_at	24.52	LTA	36092_at	26.30	PDE4DIP
36119_at	22.18	CAV1	38319_at	25.15	CD3D
35015_at	21.74	CD80	33364_at	23.68	PDE4DIP
35648_at	19.86	AUTS2	1495_at	20.36	LTBP1
35149_at	18.96	TNFRSF5/CD40	37351_at	20.09	UPP1
1983_at	18.18	CCND2	37121_at	17.04	NKG7
1319_at	11.86	DDR2	1914_at	17.00	CCNA1
32791_at	9.5	MAC30	39320_at	16.86	CASP1/caspase1
1837_at	9.31	TC21/RRAS2	34965_at	15.44	CST7/cystatin7
258_at	8.61	LTA	40520_g_at	14.42	PTPRC/CD45
40961_at	8.53	SMARCA2	38546_at	12.56	IL1RAP
34282_at	7.66	NFE2L3/NRF3	40518_at	11.28	PTPRC/CD45
40962_s_at	6.64	SMARCA2	36591_at	11.18	TUBA1
40314_at	6.44	SYNGR3	32904_at	10.68	PRF1/perforin
32640_at	6.03	ICAM1/CD54	39672_at	9.88	PTPN7
41302_at	5.73	AHCYL1	36933_at	9.08	NDRG1
37106_at	5.29	ZNF354A	40448_at	8.47	ZFP36
31888_s_at	5.28	PHLDA2/IPL	39522_at	8.29	PFKFB3
34728_g_at	4.96	AHCYL1	37111_g_at	8.02	PFKFB3
40701_at	4.8	USP13	38630_at	7.70	LASS6
32921_at	4.78	SMA4	40968_at	7.63	SOCS3
32636_f_at	4.49	SMG1	39604_at	7.59	PSCDBP/SEC7
40607_at	4.42	DPYSL2	40876_at	7.54	GYG
38459_g_at	3.99	CYB5	41523_at	7.31	RAB32
32638_s_at	3.98	SMG1	36097_at	4.44	IER2
37731_at	3.79	EPS15	41447_at	4.19	CHSY1
1785_at	3.67	JARID1A	38859_at	3.95	SEC31L2
1937_at	3.66	RB	40268_at	3.75	FOSL2
40237_at	3.65	PHLDA2			
36395_at	3.6	PRKWNK1			
38841_at	3.45	UBADC1			
34740_at	3.35	FOXO3A			
34411_at	3.33	PAPSS1			
1258_s_at	3.33	ERCC4			
41722_at	3.16	NNT			
33433_at	3.07	DKFZP564F0522			
36783_f_at	3.06	H-plk			
35150_at	3.00	TNFRSF5/CD40			
31993_f_at	2.87	CACNB2			
33891_at	2.64	CLIC4			
32401_at	2.45	NRTN			
37670_at	2.31	ANXA7			
39337_at	2.13	H2AFZ			

Transcription profiles of each of four HL cell lines were compared to four ALCL profiles in a pairwise fashion. Genes whose expression differed by at least two-fold in all 16 different comparisons are shown. Affymetrix U95 probe set ids, fold difference of the average expression signals and gene symbols of genes expressed at higher level in HL are shown on the left and of those expressed at higher level in ALCL on the right.

However, primary mediastinal B-NHL, which has been found to be related to HL[40,41] and some other B-NHL such as CD30+ diffuse large cell lymphoma or plasmablastic lymphomas were not included in this dataset. HL and ALCL samples were grouped on separate branches next to each other and were not intermingled, although one of the HL cell lines investigated is of T-cell origin (HDLM2). Thus, in these entities the cellular derivation may not determine the tumor cell phenotype. Lack of expression of most B-cell antigens but also of some common lymphoid- and T-cell antigens is one of the main features that contributed to the relatedness of HL and ALCL found in this study, which is concordant with previous studies that reported the loss of B-cell phenotype and T-cell phenotype in HL and ALCL, respectively.[14,42] However, this was not the only feature that determined the relation of HL and ALCL, since both entities still clustered next to each other when B-cell markers were excluded from the analysis. Other common expression markers of HL and ALCL can be attributed to the activated phenotype that characterizes these neoplasms, which is a well known phenomenon.[4,20] Another shared feature of HL, ALCL and LCL is transcripts that encode microfilaments and other elements of the cytoskeleton and may be linked to the morphology of the tumor cells. Of these, only fascin has been reported before in ALCL and HL,[43] whereas dystonin, myoferlin and radixin had not been previously identified. The transcription factor IER3/IEX1 was found to be expressed at higher lev-

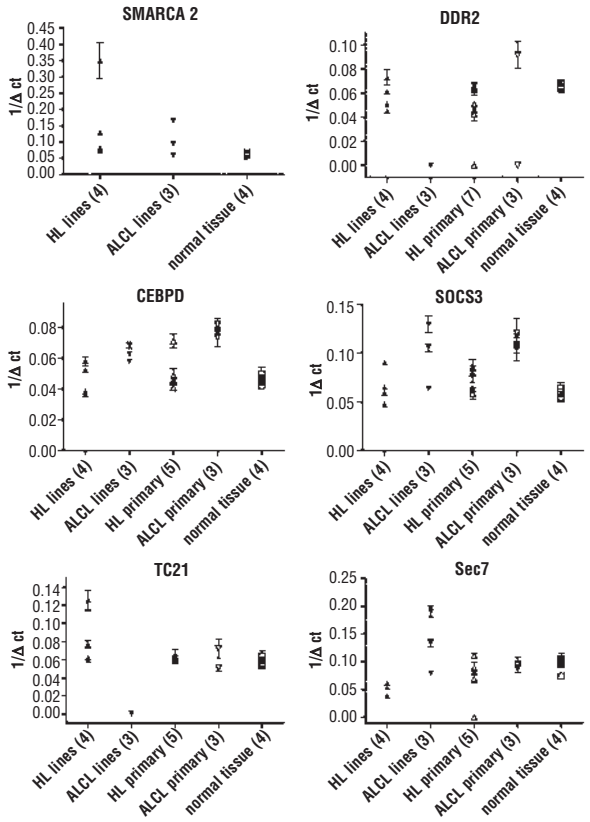

Figure 3. RT-PCR analysis of HL and ALCL for the expression of SMARCA2, DDR2, CEBPD, SOCS3, TC21, SEC7 in cell lines and primary tumor cells. Three to seven different samples/cases were investigated for each group, as indicated in brackets. For primary HL and ALCL cases 50 tumor cells were microdissected and RNA extracted from these cells was subjected to RT-PCR. Normal tissue samples were microdissected from tonsil, lymph node and germinal center and interfollicular zone of a lymph node. All reactions were carried out in triplicate. For each sample/case the mean value and the standard deviation of the three reactions are shown. The relative levels of expression are given as reciprocal values of the Δct. The ct depicts the PCR cycle when the signal of the target gene rises above a defined threshold. The Δct is the difference between the ct of the target gene and the ct of the housekeeping control gene (18s rRNA). The Δct correlates inversely with the level of expression of the target gene.

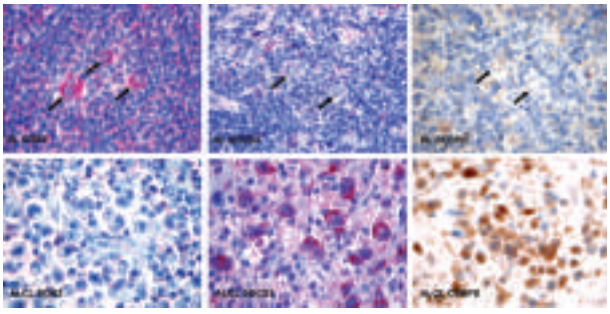

Figure 4. Immunohistochemical staining for DDR2, SOCS3 and CEBPD in HL (upper panel) and Alk+ ALCL (lower panel): The receptor tyrosine kinase DDR2 is expressed in a considerable fraction of cases of HL, but in only a few cases of ALCL; the opposite pattern is occurs for SOCS3 and CEBPD.

Table 2. Results of immunohistochemical staining of cases of primary Hodgkin's lymphoma (HL) and Alk+ anaplastic large cell lymphoma (ALCL).

	HL positive/all cases	*Alk- ALCL positive/all cases*
DDR2	17/39* (44%)	3/14 (21%)
SOCS3	11/34° (32%)	9/14 (64%)
CEBPD	1/17 (6%)	6/14 (42%)

**As previously published;[29] °as performed in a previous study.[39]*

els in HL, ALCL and LCL than in B-NHL, B-NHL cell lines and B-cell subsets in our comparison. Wu and colleagues showed that IEX1 exists in two splice variants: IEX1L and IEX1.[44] The long form, IEX1L, encodes a protein that protects cells from Fas- or TNFα-mediated apoptosis. The expression of IEX1L was shown to be dependent on NFκB activation, which occurs in HL, but not in ALCL.[45,46] Thus, IEX1L may be one further NFκB-regulated element that confers resistance to apoptosis in classical HL. Despite all the similarities of HL and ALCL described, the two entities normally originate from different cell types and show different biological and clinical behaviors. Many of the transcriptional differences of HL and ALCL identified in our study relate to well established phenomena that distinguish the two entities. In contrast to ALCL, HL tumor cells are commonly surrounded and outnumbered by a polymorphous reactive cellular background. These reactive cells are thought to be attracted by various cytokines and chemokines expressed by the HRS cells.[32,34] Indeed, we identified IL6, LTA and TARC as discriminating genes between HL and ALCL. Similarly, the expression of cytotoxic molecules is an attribute of cytotoxic T cells and we identified granzyme B and perforin as ALCL-specific mRNA. This phenomenon had already been established by immunohistochemical staining of primary ALCL and HL cases, however, in these studies the expression of cytotoxic molecules was also reported for HRS cells of a small fraction of primary HL cases.[12,38] Moreover, in most of these HL cases this cytotoxic phenotype was found to be aberrant, since only a few of these cases were T-cell-derived HL.[8,9] The genes clusterin, BCL3, IFI-56K and NM23-H4 have been described in previous comparisons of HL and ALCL as being differentially regulated.[16,18,19] Of these, only BCL3 is represented on the U95A array, which we used in our study. Higher expression of BCL3 in ALCL was not detected in our analysis, because two of the HL cell lines (L1236 and HDLM2) expressed BCL3 at comparable levels as the ALCL samples. Seven genes with possible regulatory functions or which could serve as diagnostic markers or therapeutic targets that were found to be differentially expressed in HL and ALCL in our microarray data were also investigated by RT-PCR. For three of these seven genes the difference in expression could not be confirmed, either in the cell lines (SMARCA-2) or in primary tumor tissue (SEC7, TC21/RRAS2). There is a variety of possible explanations for this: (a) the targets on the microarray may not be gene-specific but may also bind other mRNA; (b) due to the limited number of samples investigated but thousands of mRNA probed, the SMARCA-2 gene may not really be differentially expressed, but was identified because of *overfitting*;[47] (c) these mRNA

(SEC7, TC21/RRAS2) are differentially expressed in the cell lines, but not in primary tumor tissue, since cell lines are models with some peculiar gene expression patterns. We tried to overcome the last problem by investigating both cell lines and primary tumor tissue together, although this approach was limited by the lack of appropriate material. Apart from the already known differences of HL and ALCL mentioned above, some further identified genes of interest were also confirmed in primary tumor cells. The common tumor antigen PRAME has primarily been described in melanoma, but is also expressed in some lymphoid and myeloid leukemias and multiple myeloma, but rarely in B-NHL[48-50] and, as we show, not in ALCL. Recently, PRAME has been shown to be a dominant repressor of retinoic acid receptor signaling and is therefore thought to confer growth or survival advantages in cancer cells.[51] Its expression in HL may be used immunotherapeutically, since PRAME is not expressed in normal adult tissue.

In ALCL, a common transforming event has been established with translocations leading to constant activation of the receptor tyrosine kinase (RTK) Alk in a majority of ALCL cases.[4] In classical HL, approximately 40% of cases in the western world are positive for the potentially oncogenic Epstein-Barr virus,[52] but no other common oncogenic event has been established so far. Thus, it was interesting to detect the RTK DDR2 in HL, since RTK are well established oncogenes not only in ALCL.[53] Moreover, in an extended search for possible target genes in this dataset of HL cell lines, further RTK, which displayed aberrant expression in HL, were identified and their expression was confirmed in primary HRS cells by immunohistochemistry.[29] These further RTK were not detected in this study, because only genes that were expressed in all four HL cell lines were considered here, a criterion met only by DDR2. Thus, aberrant RTK signaling may be an important factor also in classical HL.[29] It is interesting to note that a fraction of primary Alk$^+$ ALCL cases investigated immunohistochemically was also positive for DDR2. Thus, apart from Alk other RTK may also be important for ALCL. SOCS proteins are potent inhibitors of many cytokine signaling cascades, including the JAK/STAT pathway.[54] Differential expression of the STAT3 regulatory gene SOCS3 has been detected in several comparisons of HL and ALCL cell lines, including ours.[17,55] However, aberrant expression of SOCS3 protein was not only detected in primary ALCL cases, but also in HRS cells of HL by immunohistochemistry.[55] Thus, although we detected an at least two-fold difference in the RNA expression of SOCS3 in primary ALCL in comparison to HL, aberrant SOCS3 expression was also found in HL. This was reflected in our microarray data, which showed a higher expression of SOCS3 in some HL cell lines as compared to B cells and B-NHL. Furthermore, the microarray data showed that another member of the SOCS-family, SOCS2, was more highly expressed in HL and ALCL than in B cells and B-NHL. Thus, SOCS proteins may confer resistance to regulatory effects of cytokines in ALCL and HL.

The CCAAT/enhancer binding protein family of transcription factors has five members: CEBPA, CEBPB, CEBPC, CEBPD, CEBPE. One of these, CEBPD also named NF-IL6β, was found to be over-expressed in ALCL cell lines and primary ALCL tumor cells in our study. CEBP transcription factors have been implicated in various physiological and pathophysiological processes, including differentiation, proliferation, inflammation and apoptosis.[56] In mice, loss of CEBPD has been shown to promote chromosomal instability.[57] Since the function of the CEBP is dependent on cell type and situation, further research is needed to establish the impact of CEBPD expression in ALCL. Taken together, our data show that: (i) at the transcriptional level HL is more closely related to Alk$^+$ ALCL than to the B-NHL and B-cell samples investigated, despite being a B-cell derived lymphoma; (ii) this relationship is to a large extent, but not exclusively, caused by down-regulation of common B-cell antigens in HL; (iii) in addition to well known differences of HL and ALCL further genes of interest identified in the transcriptome, may be important in the pathogenesis of HL and ALCL and may serve as therapeutic targets or diagnostic markers. These include the RTK DDR2 in HL, which may imply a potential role of RTK in the pathogenesis of HL, and PRAME, which could be a potential target for immunotherapy in HL. The importance of the expression of SOCS3 and CEBPD in the pathogenesis of ALCL awaits elucidation in further studies.

KW,RK: conception and design, data acquisition, analysis and interpretation, manuscript drafting and revision, final approval; CR: data acquisition, analysis and interpretation (immunohistochemistry), manuscript revision, final approval; VB, SE: data acquisition, analysis and interpretation (RT-PCR), manuscript revision, final approval; EW: data acquisition, analysis and interpretation (FACS), manuscript revision, final approval; AB: data analysis and interpretation, manuscript revision, final approval; M-LH: conception, data analysis and interpretation, manuscript revision, final approval. The authors declare that they have no potential conflict of interest.

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