Classical Hodgkin's lymphoma shows epigenetic features of abortive plasma cell differentiation

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Epigenetic changes are involved in the extinction of the B-cell gene expression program of classical Hodgkin's lymphoma. However, little is known regarding epigenetic similarities between cells of classical Hodgkin's lymphoma and plasma cell myeloma, both of which share extinction of the gene expression program of mature B cells.

Design and Methods

Global histone H3 acetylation patterns were determined in cell lines derived from classical Hodgkin's lymphoma, plasma cell myeloma and B-cell lymphoma by chromatin immunoprecipitation and subsequent hybridization onto promoter tiling arrays. H3K27 trimethylation was analyzed by chromatin immunoprecipitation and real-time DNA polymerase chain reaction for selected genes. Epigenetic modifications were compared to gene expression data.

Results

Characteristic B-cell genes were hypoacetylated in classical Hodgkin's lymphoma and plasma cell myeloma cell lines as demonstrated by comparison of their histone H3 acetylation patterns to those of B-cell lines. However, the number of genes jointly hyperacetylated and expressed in classical Hodgkin' lymphoma and plasma cell myeloma cell lines, such as *IRF4/MUM1* and *RYBP*, is limited. Moreover, H3K27 trimethylation for selected characteristic B-cell genes revealed that this additional epigenetic silencing is much more prevalent in classical Hodgkin's lymphoma than in plasma cell myeloma.

Conclusions

Our epigenetic data support the view that classical Hodgkin's lymphoma is characterized by abortive plasma cell differentiation with a down-regulation of characteristic B-cell genes but without activation of most genes typical of plasma cells.

Key words: epigenetics, acetylation, Hodgkin's lymphoma, B-cell lymphoma, multiple myeloma.

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Introduction

Disruption of epigenetic patterns is recognized as one of the hallmarks of cancer and plays a central role in determining the tumor phenotype.[1] Furthermore, epigenetic analyses are of interest since epigenetic therapy is emerging as a valuable and effective treatment approach for peripheral T-cell lymphomas and is also envisaged for B-cell and Hodgkin's lymphomas.[2-5]

There are different sets of epigenetic instructions involved in the regulation of gene expression.[1] DNA methylation of gene promoters contributes significantly to gene silencing whereas histone modifications such as acetylation of lysine 9 and 14 of histone H3 (H3K9/14 acetylation) are positively correlated with gene activation.[1] Furthermore, epigenetic silencing of genes can be mediated by Polycomb group (PcG) proteins.[6] PcG proteins were first identified in Drosophila as regulators of the expression of *Hox* genes.[7] In humans the PcG proteins comprise two functionally and biochemically distinct multimeric Polycomb repressive complexes, called PRC1 and PRC2. According to a currently proposed model PRC2 initiates transcriptional repression through trimethylation of histone H3 lysine 27 (H3K27) whereas PRC1 maintains this repressive condition.[6] PcG complexes play an important role in B-cell development and the germinal center reaction, and increased or decreased activity of PcG proteins is thought to contribute to lymphomagenesis.[8-10] The precise interplay between the various epigenetic mechanisms is very complex and not fully understood.[1,11,12]

Classical Hodgkin's lymphoma (cHL) is a monoclonal lymphoid neoplasm derived from (post-) germinal center B cells in almost all instances.[13-15] Morphologically, cHL is composed of a usually small number of mononuclear Hodgkin (H) cells and multinucleated Reed-Sternberg (RS) cells residing in an extensive inflammatory background.[15] A key feature, that distinguishes cHL from other B-cell lymphomas is the almost complete absence of B-cell markers from the HRS cells (e.g. CD19, CD20 and CD79b) and the up-regulation of B-cell lineage inappropriate genes.[15,16]

Recently it has been proposed that the initial events that finally lead to cHL are caused by epigenetic modifications which have the capacity to induce a transcriptional avalanche effect including up-regulation of cHL-characteristic but B-cell lineage inappropriate genes and extensive down-regulation of the B-cell expression program.[17]

Down-regulation of genes typically expressed in mature B-cells also occurs during the course of plasma cell differentiation, suggesting that HRS cells might be related to plasma cells.[18,19] However, immunoglobulin gene expression - which is extremely high in plasma cells - is completely absent from HRS cells. In addition most other markers typically up-regulated in plasma cells are not produced by HRS cells.[18]

To elucidate the extent of the relationship between HRS cells and plasma cells from an epigenetic perspective, we analyzed their genome-wide histone H3 acetylation pattern in corresponding cell lines and compared the results with those obtained from B-cell lines. For this genome-wide analysis high density promoter tiling arrays were used in combination with chromatin immunoprecipitation (ChIP-on-chip) employing H3K9/14ac antibodies. Although *in-vitro* cultured cancer cells might contain some artificial epigenetic modifications, we employed established cHL, B-cell lymphoma and plasma cell myeloma (PCM) cell lines since these have retained their cell type-specific phenotype and are thus useful models reflecting the *in-vivo* situation in many aspects.[20] Our results provide evidence that cHL is derived from cells with incomplete plasma cellular differentiation that show extensive down-regulation of B-cell antigens but no activation of most plasma cell typical genes. Furthermore, additional suppressive H3K27 trimethylation of B-cell characteristic genes is more extensively found in cHL than in PCM cell lines.

Design and Methods

Cell cultures

Three cHL cell lines (L1236, KM-H2, L428), three PCM cell lines (L363, U266, LP-1, referred to as PCM cell lines) and four B-cell lymphoma cell lines [SU-DHL4, SU-DHL6, HT (diffuse large B-cell lymphoma; DLBCL) and Namalwa (Burkitt's lymphoma; BL) (referred to as B-cell lines] were cultured with 5% CO_2 in RPMI 1640 (PAA, Pasching, Austria) and supplemented with 10% fetal bovine serum (PAA) at 37°C.

Chromatin immunoprecipitation

B-cell lines (SU-DHL4, SU-DHL6, Namalwa), cHL cell lines (L1236, KM-H2, L428) and PCM cell lines (L363, U266, LP-1) were used for chromatin immunoprecipitation (ChIP) following the protocol developed by Young's group with minor modifications.[21] For each ChIP experiment 10 μg of anti-acetyl-histone H3 (Lys9 + Lys14) antibody (06-599; Millipore, Temecula, CA, USA) were employed. In addition, H3K27 trimethylation was analyzed by ChIP for selected genes in cell lines derived from cHL (L428, L1236, KM-H2), PCM (U266, L363) and B-cell lymphomas (Namalwa, SU-DHL4) using 15-20 μg of an anti-H3K27 trimethylation antibody (07-499, Millipore). The specificity and suitability of these antibodies for ChIP were demonstrated in a previously published study.[17,22,23] Details of the protocol are provided in *Online Supplementary Protocol S1*.

Successful enrichment of ChIP DNA-fragments was confirmed by quantitative real-time polymerase chain reaction (PCR) employing SYBR Green PCR-Master Mix (Applied Biosystems, Foster City, CA, USA) on a GeneAmp7900HT Fast Real-Time PCR system (Applied Biosystems).[17,24] Primer sequences are listed in *Online Supplementary Table S1*.

Histone H3 acetylation ChIP-on-chip hybridization and data analysis

The workflow of the entire ChIP-on-chip approach is depicted in Figure 1. The GeneChip® Human Promoter 1.0R Tiling Array (Affymetrix, Santa Clara, CA, USA) enables determination of protein/DNA interactions for over 25,500 human promoters which are densely covered by oligonucleotides covering approximately 7.5 kbp upstream and 2.45 kbp downstream of 5' transcription start sites. For over 1,300 cancer-associated genes, the upstream promoter coverage is extended to 10 kbp.

Approximately 250 ng of ChIP-DNA were used as a template for ligation-mediated linear amplification according to the protocol of Young *et al.* to obtain a sufficient amount of labeled DNA for chip hybridization.[21] The GeneChip WT double-stranded DNA terminal labeling kit (Affymetrix) was used for DNA-fragmentation and labeling according to the manufacturer's instructions. Reaction mixtures were hybridized to the chips for 16 h at 45°C at 60 rpm and stained with streptavidin/phycoerythrin, followed by a biotin-conjugated anti-streptavidin antibody and a second streptavidin/phycoerythrin staining. All liquid handling was carried out by a GeneChip Fluidics Station 450. GeneChips

were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix) and CEL files were generated with GCOS 1.3 software (Affymetrix). All experiments were performed in triplicate (cHL and B-cell lines) or duplicate (PCM cell lines). The CEL files of all experiments are available via the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (*www.ncbi.nlm.nih.gov/geo/*) under the accession number GSE21254.

The resulting CEL files were analyzed using the model-based analysis of tiling arrays (MAT) algorithm.[25] We used the default adjustments except for the MaxGap parameter which was reduced to 50 bp and the *P* value which was set at 0.005 to find acetylated regions at higher resolution. Instead of working with the original Affymetrix BPMAP files, we used the microarray probe mapping to NCBI build 36 provided by the MAT homepage (http://liulab.dfci.harvard.edu/MAT/). This analysis resulted in a list of significantly acetylated regions for each of the nine cell lines. The most significantly acetylated 11,000 regions per cell line were selected for further analysis.

Our analysis was primarily focused on the identification of acetylated genes. We, therefore, removed from the full list of 46,875 Entrez genes those that lack a validated or reviewed entry in RefSeq, leading to a list of 18,546 Entrez genes associated with 24,885 RefSeq entries. The RefSeq collection includes alternatively spliced transcripts and information about usage of different transcription start sites (TSS).

We calculated a cell line-specific acetylation score for each RefSeq entry as follows: all acetylated regions in a genomic window between 10 kbp upstream of the TSS and 2.45 kbp downstream of the TSS were determined. This window size was set according to the coverage of the promoter tiling array. All acetylation signals within this region were aggregated to an overall score. Longer acetylated regions therefore got a higher score than did shorter regions. Furthermore, since acetylated regions inside the core promoter region are expected to have greater relevance for gene expression, we over-weighted such regions 2-fold. Core promoters were defined as 500/100 bp up/down-stream of the TSS, referring to models employed by the Genomatix software tool (Munich, Germany).[26]

For the detection of genes specifically acetylated in cHL and PCM cell lines, as compared to in B-cell lines, we calculated a *P* value for each RefSeq region using a moderated t-test. This *P* value is a measure of the probability that the observed difference in acetylation between two types of cell lines occurs by chance.[27] For all genes with more than one RefSeq sequence, we retained the RefSeq sequence with the best *P* value. We call a gene specifically acetylated if its *P* value is less than 0.05. We also computed an acetylation fold change. Further details are given as supplementary information (*Online Bioinformatic Algorithm S1*).

Enrichment analysis of biological annotations

The Entrez gene identifiers of differentially acetylated genes were uploaded to DAVID (database for annotation visualization and integrated discovery, http://david.abcc.ncifcrf.gov/).[28,29] We calculated the most over-represented (enriched) biological annotations employing DAVID default parameters and using the 18,546 Entrez gene identifiers covered by the ChIP-on-chip analysis (*Online Supplementary Table S2*) as the background distribution.

Combined 5-aza-2´-deoxycytidine and trichostatin A treatment

The DLBCL-derived cell lines SU-DHL4, SU-DHL6 and HT were demethylated and acetylated by treatment with 5-aza-2´-deoxycytidine (AZA; Sigma-Aldrich, St. Louis, MO, USA) and trichostatin A (TSA; Sigma, Steinheim, Germany) according to established protocols.[17] AZA was applied at a concentration of 3 μM for 6 days with replacement on days 2 and 5. On day 5, cells were additionally incubated for 24 h with 625 nM TSA and harvested on day 6. The data from these cell lines were analyzed in combination with data already available for AZA/TSA-treated BL-derived cell lines Raji, Daudi and Namalwa derived from our previous study (GEO accession number GSE8388).[17]

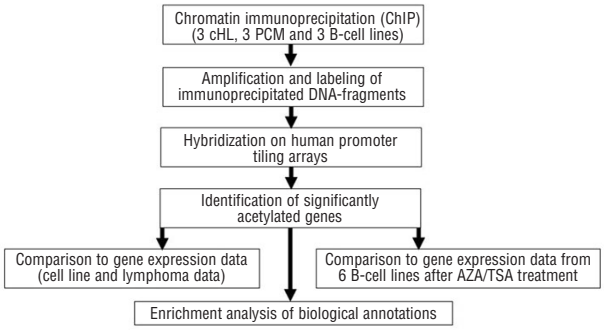

Figure 1. Workflow for the analysis of acetylation patterns and gene expression in cHL, PCM and B-cell lines.

Generation and analysis of gene expression data

Gene expression analysis was performed for each of the three PCM cell lines. Furthermore, gene expression data were generated in duplicate for the B-cell lines SU-DHL4, SU-DHL6 and HT, which were treated with AZA and TSA as described above. For this purpose, RNA was isolated according to standard protocols (Qiagen, Hilden, Germany) and GeneChip hybridization was carried out with Affymetrix GeneChips HG-U133A using 5 μg high quality total RNA according to the manufacturer's recommendations.

In addition publically available expression data from our group were used (cHL cell lines [KM-H2, L1236, L428] and B-cell lines [Raji, Namalwa, Daudi]; GEO accession number GSE8388).[17] Gene expression data were analyzed using the statistical programming environment R and Bioconductor after normalization using Robust Multichip Average (RMA). Fold changes were determined[27] and *P* values were calculated using a moderated t-test with subsequent Benjamini-Hochberg (BH) correction.[30] We defined a probe set as significantly differentially expressed if: (i) at least a 2-fold change was observed and (ii) the BH-corrected *P* value was below 0.05. Probe sets were mapped to Entrez gene identifiers using the BioMart service and library.[31,32] If more than one probe set was associated with a specific gene, only the probe set with the lowest *P* value was used.

Based on the Entrez gene identifiers, the results of the differential acetylation analysis and the expression analysis were merged. To compare the ChIP-on-chip data with the results obtained from the gene expression arrays, we generated Venn diagrams. Furthermore published gene expression data from microdissected tumor cells of 12 cHL cases and 21 B-cell lymphoma cases [11 DLBCL, 5 BL and 5 follicular lymphoma (FL)] cases (available through GEO accession number GSE12453)[33] were used to generate Venn diagrams for independent comparison with the differentially acetylated genes in cHL and B-cell lines. A single-sided Fisher's exact test was applied to calculate the significance of the intersections in the Venn diagrams. We also generated a heat map using GeneChip HG-U133A expression data from cHL, PCM and B-cell lines employing a list of 158 differentially acetylated genes in cHL and PCM cell lines as compared to in B-cell lines.

Results

Analysis of histone H3 acetylated genomic intervals in classical Hodgkin's lymphoma, plasma cell myeloma and B-cell lines

ChIP employing a H3K9/14ac-specific antibody and subsequent hybridization of the DNA-fragments to promoter tiling arrays (ChIP-on-chip) was able to identify numerous acetylated genomic intervals in each cell line. Real-time DNA-PCR for selected genes revealed an excellent correlation with the data derived from the ChIP-on-chip experiments (*Online Supplementary Figure S1*). The boxplot for the lengths of the selected genomic ChIP-on-chip intervals for each cell line (11,000 most significant intervals per cell line) showed comparable results (*Online Supplementary Figure S2*).

In order to generate cell type-specific acetylation patterns we compared the ranked list of acetylated genes obtained in B-cell lines with those derived from cHL and PCM cell lines (*Online Supplementary Table S2*). The comparison of cHL and B-cell lines revealed that 211 genes were specifically acetylated in cHL (cHL_{Ac}) and 327 ($B1_{Ac}$) in B-cell lines (Figure 2). The consideration of B-cell and PCM cell lines led to the identification of a higher number of genes specifically acetylated in B cells (n=591; $B2_{Ac}$) and 143 genes were specifically acetylated in PCM cell lines (PCM_{Ac}) (Figure 2).

As expected, the overlap of the two sets of genes specifically acetylated in B-cell lines ($B1_{Ac}$ and $B2_{Ac}$) was high (n=141) and comprised well known typical B-cell genes (e.g. *CD19*, *CD79a/b*, *BLNK*). Vice versa, these 141 genes can be regarded as being hypoacetylated in cHL as well as in PCM cell lines. In contrast, the intersection of the specifically acetylated genes in cHL and PCM cell lines revealed a much lower but highly significant number of genes (n=17). Interestingly, these 17 genes included *IRF4/MUM1*, which is known to be consistently expressed in cHL and PCM cells.[34,35] In order to generate a list of genes highly specifically acetylated exclusively in cHL cell lines we removed these 17 genes from cHL_{Ac} which resulted in 194 genes. The color code corresponding to the various intersections in Figure 2 was also applied to the respective genes in *Online Supplementary Table S3*.

Enrichment analysis of biological annotations

To gain more insight into the biological function of the significantly acetylated or hypoacetylated genes in cHL and PCM cell lines we calculated the most over-represented (enriched) annotations using the DAVID tools.[28,29] The 211 genes acetylated in cHL cell lines were enriched for genes involved in the regulation of apoptosis and cell death, myeloid differentiation and the Toll-like receptor pathway whereas the 143 genes specifically acetylated in PCM cell lines were enriched for cAMP-mediated signaling and genes related to transcriptional repression. The only term enriched in the 17 genes specifically acetylated in both cHL and PCM cell lines was "ATP binding" (*Online Supplementary Table S4*). Finally, the 141 genes present in both B-cell lists ($B1_{Ac}$ and $B2_{Ac}$) were dominated – as expected - by terms related to the B-cell receptor signaling and immune response (*Online Supplementary Table S4*).

Comparison of gene expression and histone H3 acetylation status

To verify the functional impact of the epigenetic profiles assessed by H3K9/14ac ChIP-on-chip, the transcriptional activity of the differentially acetylated genes was analyzed. The linkage of Entrez gene identifiers of the acetylated genes to gene expression (Affymterix HG-U133A) revealed a highly significant positive correlation between gene expression and histone H3 acetylation (*Online Supplementary Figure S3*). The strong positive correlation between histone H3 acetylation and gene expression is demonstrated in a heat map based on 158 genes differentially acetylated in cHL and PCM cell lines (17 hyperacetylated and 141 hypoacetylated genes; Figure 3). This clear positive correlation is also exemplarily shown [*CD30*, *CCR7*, *IRF4* and *PRDM1/BLIMP1*, *CD20* (*MS4A1*), *CD79a*, *BOB1* (*POU2AF1*)] in *Online Supplementary Figure S4*.

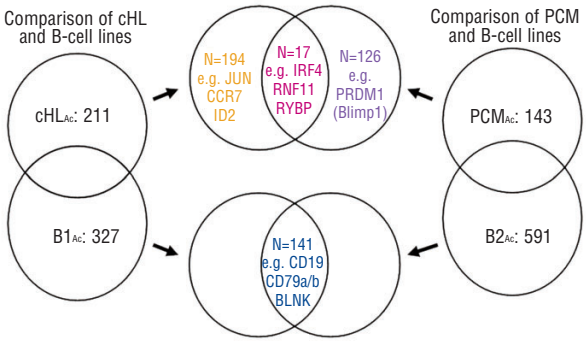

Figure 2. The genes acetylated in cHL, PCM and B-cell lines were compared to each other in order to identify genes differentially acetylated in the respective groups. Since the comparison of genes acetylated in B-cell lines and cHL or PCM cell lines, respectively, represents two different intersections, two different lists of differentially expressed B-cell genes ($B1_{Ac}$ and $B2_{Ac}$) were generated. Some examples of cell type-characteristic genes are mentioned in the intersections. The corresponding gene lists are given in *Online Supplementary Table S3*.

To estimate the relevance of our epigenetic study in cell lines for the *in-vivo* situation, differentially acetylated genes in cHL and B-cell lines were additionally compared to published gene expression data derived from micro-dissected tumor cells of 11 cHL cases and 21 B-cell lymphoma cases (11 DLBCL, 5 BL and 5 FL).[33] Remarkably, the comparison of these completely independent data sets also revealed highly significant overlaps. Thirty-one genes up-regulated in primary HRS cells were also acetylated in cHL cell lines (P =1.950429e-24) and 65 genes found to be expressed in primary DLBCL, BL and FL cells (P =8.810192e-37) displayed an acetylation in the B-cell lines (*Online Supplementary Figure S5* and *Online Supplementary Table S5*). No overlapping genes were observed in any of the other intersections.

Comparison of 5-aza-2´-deoxycytidine and trichostatin A induced differential gene expression and histone H3 acetylation

Previous work by our group demonstrated that the epigenetic treatment of B-cell lines with AZA (DNA demethylation) and TSA (histone acetylation) led to an almost complete extinction of their B-cell identity and up-regulation of B-cell-inappropriate cHL-characteristic genes.[17] We were, therefore, interested in correlating the ChIP-on-chip acetylation data with the gene expression induced by AZA and TSA treatment of B-cell lines. For this purpose we supplemented our previously published data with data from additional AZA/TSA-treated DLBCL cell lines (SU-DHL4, SU-

DHL6, HT) giving rise to a final data set from six different B-cell lines. Overall, 2230 probe sets were shown to be significantly affected by the treatment when all treated and untreated cell lines were compared. The majority of these transcripts (1671) were down-regulated whereas only 559 were up-regulated by the treatment. Since several probe sets cover the same gene, we mapped all probe sets to the corresponding Entrez gene identifiers leading to 1236 down-regulated and 472 up-regulated genes. From these, 1194 down-regulated and 435 up-regulated genes were present on the promoter tiling arrays.

Subsequently, the data derived from AZA/TSA-induced differential gene expression in B-cell lines were compared to the data obtained from the ChIP-on-chip acetylation analysis (*Online Supplementary Figure S6*). Genes specifically acetylated in B-cell lines and down-regulated by AZA/TSA treatment of B-cell lines displayed an overlap of 67 genes (P=6.047922e-16) including characteristic B-cell transcripts such as *CD19*, *CD20* and *CD79a/b* (*Online Supplementary Table S6*). The comparison of the genes specifically acetylated in HRS cells and the genes up-regulated upon epigenetic treatment of B-cell lines identified 22 genes (P=1.452767e-07) in common (*Online Supplementary Table S6*). The remaining comparisons were not statistically significant (*Online Supplementary Figure S6*).

Analysis of Polycomb group-mediated H3K27 trimethylation for selected genes

Quantitative real-time PCR with DNA obtained after ChIP with antibodies against H3K27 trimethylation revealed that the promoters of ten selected characteristic B-cell genes (*CD19*, *CD20* (*MS4A1*), *CD79b*, *BOB1* (*POU2AF1*), *PU.1*, *SYK*, *LCK*, *TCL1A*, *BCMA*, *PAX5*) were predominantly enriched in the cHL cell lines (Figure 4). In contrast, promoters of genes usually highly expressed in cHL cell lines (*CD30*, *CCR7*, *TRAF1*, *SEMA4C*, *IL6*) showed no enrichment after ChIP for H3K27 trimethylation with the exception of *CD30* which is known to be expressed in the cHL cell line L1236 at a very low level. An inverse H3K27 pattern was found in the B-cell lines confirming the gene silencing function of this histone trimethylation. Interestingly, H3K27 trimethylation and H3K9/14 hypoacetylation were detectable in the *CD20* promoter of Namalwa cells which is, however, well in line with its strongly reduced *CD20* mRNA expression (*Online Supplementary Figure S4*). Finally and most strikingly, only *PAX5* and three other B-cell genes (*TCL1A*, *CD20*, *SYK*) were additionally silenced by means of H3K27 trimethylation in PCM cell lines. In contrast, the majority of B-cell genes in cHL cell lines showed a repressive H3K27 trimethylation and H3 hypoacetylation (Figure 4).

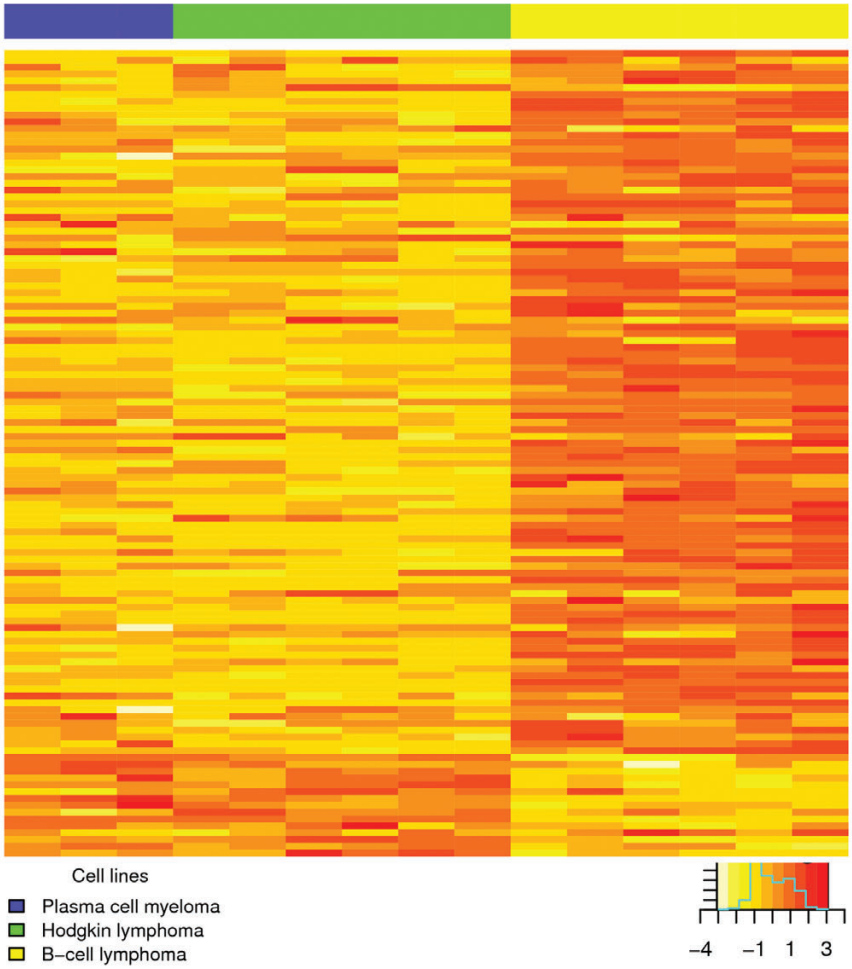

Figure 3. The genes used for hierarchical cluster analysis were selected exclusively based on their acetylation pattern. For this purpose, genes acetylated in cHL and PCM cell lines (n=17) and the 141 significantly acetylated genes derived from the B-cell lists $B1_{Ac}$ and $B2_{Ac}$ were selected (Figure 2). To assess their gene expression, the list of acetylated genes was linked to gene expression data derived from completely independent Affymetrix gene chip (U133A) analyses of the corresponding cHL, PCM and B-cell lines.

Discussion

The biological events that initiate the almost complete extinction of the B-cell phenotype and transformation of cHL are still unknown.[20] Several explanations for this phenomenon have been proposed, including a defective transcription factor machinery responsible for the down-regulation of the respective B-cell antigens.[20,36,37] Another possible mechanism, namely down-regulation of the B-cell phenotype as in plasma cells, has been considered only rarely[18,19] although there are several arguments that support this scenario (e.g. immunohistological expression of IRF4 or late class switch recombination events in cHL cell lines).[18,34,35,38]

To explore the relation of cHL and plasma cells at the epigenetic level, we investigated the global histone H3 acetylation pattern in cHL and PCM cell lines and, for comparison, in several B-cell lines. These acetylation data were supplemented with the H3K27 trimethylation status for selected genes and the functional impact of these epigenetic patterns on the transcriptome was determined.

Analysis of acetylated regions in classical Hodgkin's lymphoma, plasma cell myeloma and B-cell lines

ChIP with subsequent hybridization of the precipitated DNA-fragments to promoter tiling arrays (chip) was performed to identify the global acetylation pattern of cHL, PCM and B-cell lines. After determination of genes specifically acetylated in each cell line group, we compared these cell type-characteristic patterns to each other. This led to the identification of 141 genes which were commonly hypoacetylated in cHL and PCM cell lines and hyperacetylated in B-cell lines (Figure 2). Not surprisingly, 23 of these genes were related to B-cell receptor signaling or immune response (*Online Supplementary Table S4*) which is compatible with their consistent down-regulation in cHL and PCM. In contrast, the number of genes acetylated in both cHL and PCM cell lines, but not in B-cell lines, was much smaller (n=17). Moreover, genes specifically acetylated only in cHL cell lines (n=194) were frequently involved in cell death, Toll-like receptor (TLR) signaling pathway and myeloid differentiation. This fits very nicely with the observations that: (i) HRS cells are prone to apoptosis,[20] (ii) the TLR pathway might be associated with cHL pathogenesis[39] and (iii) HRS cells frequently express myeloid markers.[20]

These epigenetic patterns reveal that cHL and PCM cell lines are very similar with respect to the hypoacetylation of B-cell-characteristic genes, a finding which might explain the extinction of their B-cell identity. However, the cHL and PCM cell lines only share a very limited number of hyperacetylated genes and genes essential for complete plasma cell differentiation. Although *IRF4*, a transcription factor known to be important for plasma cell differentiation, is present among these 17 genes, another gene essential for plasma cell development (*PRDM1/BLIMP1*) was hyperacetylated only in the PCM cell lines but not in the cHL cell lines (Figure 2).[19] The latter finding, in particular, is compatible with the observation that HRS cells are unable to express genes characteristic of plasma cells.[18]

The impact of our epigenetic patterns on the transcriptome was estimated by correlation of the acetylated genes with their corresponding gene expression as assessed by Affymetrix GeneChip analysis. This revealed a strong positive correlation and clearly demonstrates that histone H3 acetylation affects gene expression to a large extent. However, acetylation as well as other epigenetic mechanisms (e.g. DNA methylation and H3K27 trimethylation) do not reflect an on/off situation but represent modulators able to control gene expression quantitatively. In addition, other non-epigenetic mechanisms (e.g. transcription factor activity) are necessary to induce and to maintain gene expression. It is not, therefore, surprising that a significant number of acetylated genes are - despite open chromatin - not concurrently up-regulated in their gene expression.

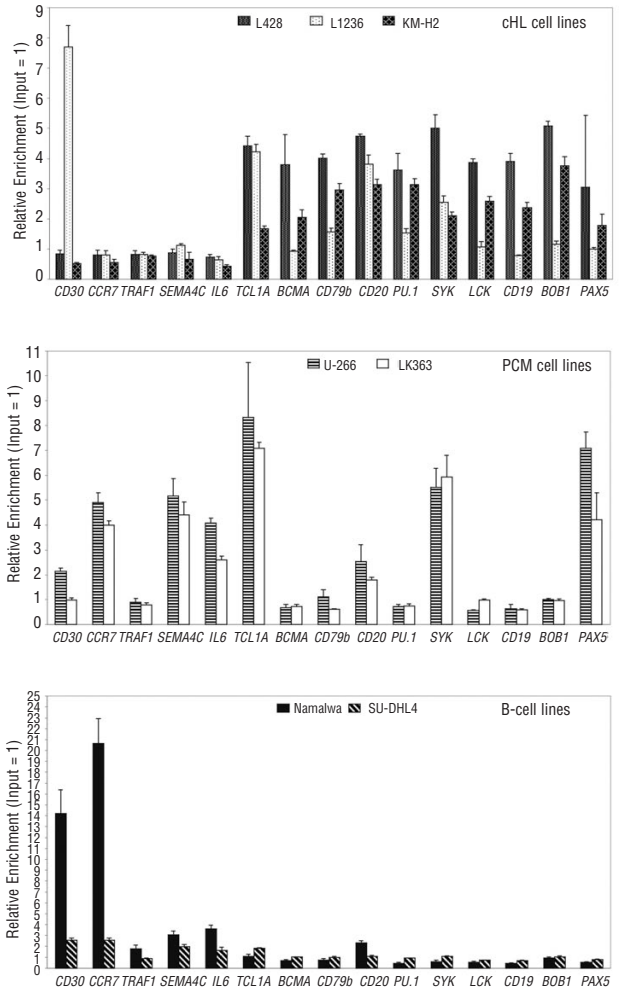

Figure 4. Promoter H3K27 trimethylation status of B-cell- and cHL-characteristic genes after ChIP and quantification by real-time DNA-PCR in cHL, PCM and B-cell lines. β-actin was chosen as the endogenous reference and the enrichment was calculated relative to the ChIP input control. ChIP: chromatin immunoprecipitation.

H3K9/14 histone acetylation negatively correlates with the DNA-methylation status in characteristics B-cell genes

It is well known that there is cross-talk between the different epigenetic mechanisms.[11] To elucidate this aspect for characteristic B-cell genes in cHL we analyzed the H3K9/14 acetylation pattern with respect to DNA methylation. For this purpose we selected nine B-cell-specific promoters

[*CD19*, *CD20* (*MS4A1*), *CD79b*, *POU2AF1*(*BOB1*), *PU.1* (*SPI1*), *SYK*, *LCK*, *TCL1A*, *BCMA* (*TNFRSF17*)] known to be silenced by DNA hypermethylation in cHL based on published data.[40,41] With the exception of *TCL1A*, *CD20* and *BCMA*, all promoters investigated displayed an additional histone deacetylation in cHL cell lines thus preventing the binding of transcription factors and, in consequence, the expression of the respective genes. The promoters of *CD19*, *CD79b*, *PU.1* and *SYK* were also found to be hypoacetylated in PCM cell lines, consistent with the absent expression of these genes (*Online Supplementary Figure S7*). Strikingly and expected, *BOB1* (*POU2AF1*), a transcription factor essential for immunoglobulin gene expression was significantly acetylated in PCM cell lines but not in cHL cell lines (*Online Supplementary Figure S4*).

Our findings suggest that the cooperative interplay between acetylation and methylation is also effective in silencing genes in cHL and PCM. The results of previous studies describing a reactivation of B-cell genes in cHL cell lines exclusively by simple demethylation are in conflict with this concept.[41] However, thorough reinvestigation of these reported demethylation effects by real-time reverse transcriptase PCR revealed merely a very weak induction of gene expression thus questioning the biological significance of the previous findings.[17,41] Interestingly, combined demethylation and acetylation is also unable to push the cHL cell lines towards a B-cell phenotype.[17] This clearly demonstrates that histone acetylation and DNA demethylation alone are not sufficient to restore the B-cell phenotype in cHL cell lines. Other mechanisms, such as inhibition of B-cell-specific transcription factors, must also be involved.[42]

Epigenetic networking in classical Hodgkin's lymphoma

As previously demonstrated, genes atypically up-regulated in cHL are especially important for subsequent extinction of the B-cell phenotype and – potentially – for the pathogenesis of cHL.[42] To identify the most relevant genes important for a better understanding of the cHL biology under an epigenetic perspective we compared: (i) genes up-regulated by AZA/TSA treatment of B-cell lines and (ii) genes specifically acetylated in cHL cell lines (*Online Supplementary Figure S6*). Twenty-two genes fulfilled these criteria. Strikingly, several of these genes, such as *ATF3*, *JUN*, *IRF4*, *ID2*, *FSCN1*, *CCR7* and *RYBP* have already been recognized by previous completely independent studies and modifications of these genes are suggested to play a role in the pathogenesis of cHL.[20,42-45]

Our special attention was attracted by the Ring1 and YY1 binding protein (RYBP) due to its importance as an interacting partner for the Polycomb group protein Ring1A.[46] Polycomb group (PcG) proteins act as transcriptional repressors by means of histone modification and are involved in the regulation of organogenesis and cell lineage fidelity.[6]

RYBP was shown to be over-expressed in primary cHL cases and to have prognostic relevance whereas it is not detectable in normal B cells of lymphoid tissue.[45,47] Although the precise mechanism of RYBP activity is not known it is likely that its up-regulation in HRS cells contributes significantly to epigenetic silencing of the B-cell phenotype. This view is supported by the fact that not only RYBP was found to be up-regulated in HRS cells but also other components of the multimeric polycomb repressive complexes such as RING1, BMI1, MEL-18, EED and EZH2.[48] These findings, together with the results obtained from our global acetylation analysis, led us to conclude that a derailed epigenetic network is involved in the extinction of the B-cell program and in the malignant transformation of cHL.

This view is additionally supported by our H3K27 trimethylation analysis of ten B-cell promoters (*CD19*, *CD20*, *CD79a*, *BOB1*, *PU.1*, *SYK*, *LCK*, *TCL1A*, *BCMA*, *PAX5*) which demonstrated an almost inverse pattern in the cHL cell lines as compared to the B-cell lines. (Figure 4). Interestingly, the H3K27 trimethylation patterns of PCM cell lines differed to some extent from those of the cHL cell lines. Whereas B-cell characteristic genes such as *CD19* and *CD79a* showed a suppressive H3K27 trimethylation in cHL, but not in PCM cell lines, *PAX5*, a master regulator of the B-cell expression program, was H3K27-trimethylated in both cell line groups. This shared epigenetic modification of the *PAX5* gene reinforces the hypothesis that PCM and cHL cell lines harbor a common molecular basis for the silencing of the B-cell expression program which is more pronounced in cHL than in PCM. Thus PcG-mediated H3K27 trimethylation can be regarded as a kind of failsafe mechanism to ensure that gene silencing by deacetylation and promoter DNA methylation is additionally stabilized in cHL. Furthermore, the additional trimethylation of H3K27 appears to be of special interest for permanent silencing of the B-cell phenotype in cHL, since this epigenetic modification is not directly affected by AZA/TSA treatment.

Taken together, our results demonstrate on a global scale that histone H3 deacetylation contributes significantly to the almost complete extinction of the B-cell expression program of cHL. This acetylation-mediated silencing is further reinforced by H3K27 trimethylation (this study) and DNA promoter methylation (published data). With respect to the hypoacetylation-mediated down-regulation of the B-cell expression program, PCM cell lines very closely resemble cHL cell lines. However, PCM and cHL cell lines differ in their hyperacetylation and H3K27 trimethylation patterns, a fact in line with the fundamental difference of genes expressed in the two entities. Thus, our epigenetic data support the view that cHL is characterized by an abortive plasma cell phenotype with down-regulation of characteristic B-cell genes but without activation of most genes typical of plasma cells.

Authorship and Disclosures

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