ETS1 encoding a transcription factor involved in B-cell differentiation is recurrently deleted and down-regulated in classical Hodgkin's lymphoma

The neoplastic B-cell derived Hodgkin and Reed-Sternberg cells (HRS) in classical Hodgkin's lymphoma (cHL) undergo a substantial loss of B-cell identity. It has been suggested that epigenetic mechanisms such as DNA methylation are involved in this cHL-associated reprogramming.[1] In a recent array-based DNA-methylation study using 27K methylation arrays, we identified 209 protein encoding genes hypermethylated in cHL but not in other germinal center derived B-cell lymphomas as compared to normal B cells. These genes, exclusively hypermethylated in cHL, are enriched for functions such as regulation of B-cell or T-cell activation.[2]

DNA methylation is an epigenetic mechanism associated with gene inactivation that can not only cause gene silencing *de novo*, but can also be acquired as a consequence of upstream gene downregulation[3] and in cHL, a loss of a master transcription factor could lead to the methylation of genes involved in B-cell identity. We, therefore, tested the 209 genes hypermethylated exclusively in cHL that we had identified in our previous study for transcription factor binding sites.[2] The most significant enrichment was seen for the transcription factors EBF1/OLF1 and E2A (E12/E47) which have both been previously shown to be involved in B-cell regulation and to be deregulated in cHL (Table 1).[4-5] Binding sites for another key B-cell transcription factor, ETS1, ranked third among the group of genes hypermethylated exclusively in cHL. This led us to analyze the *ETS1* gene for mutations, copy number alterations and expression both on mRNA and protein level in cHL cell lines and primary biopsies.

We collected published gene expression microarray data for the L428, HDLM2, KMH2 and L1236 cHL cell lines as compared to normal B-cell entities (5 centroblasts, 5 centrocytes, 5 naive B cells, 5 memory B cells)[6] and analyzed the expression of the transcription factors that were identified (Table 1). ETS1 was strongly down-regulated with a 7.23-fold reduced expression in cHL cell lines (t-test $P<0.001$) (Figure 1A). This prompted us to analyze whether the observed downregulation of *ETS1* is also present in primary cHL and results in reduced protein expression. In reactive tonsils, the nuclei of germinal center, mantle zone and parafollicular cells expressed the ETS1 protein as shown by immunohistochemistry whereas the squamous epithelium remained negative. In 8 of 17 (47%) evaluable cHL, the HRS cells were completely devoid of ETS1 protein expression whereas expression was confirmed in the nuclei of bystander cells (Figure 1B). In addition, 7 of 17 (41%) cHL presented a heterogeneous pattern of expression with both positive and negative HRS cells for ETS1 protein. Only in 2 of 17 (12%) cases did all HRS cells retain expression of ETS1 on a protein level.

As *ETS1* was not among the hypermethylated genes in cHL and, therefore, is itself not silenced by gene methylation,[2] we performed mutation screening for the entire coding sequence of this gene in 7 cHL cell lines. In the L540 cell line, an 11 bp heterozygous intronic deletion (NM_001143820.1:c.1123+5_1123+15delGCGCCCTCCCG; chr11:128,350,071-128,350,081; hg19) was identified. This deletion cannot be attributed to a known copy number variation as only a single nucleotide polymorphism (SNP) (chr11:128350071) is annotated for this location according to the University of California Santa Cruz (UCSC) Genome hg19 (*http://genome.ucsc.edu*) and the 1000 Genome (*http://browser.1000genomes.org*) databases. As the deletion is located 5 bp from the 3' splicing site of exon 8, we used RT-PCR for exons 6-10 to detect potential changes in splicing but this mutation was not seen to exert any effect. These findings suggest that the deletion identified does not affect ETS1 protein functionality. Neither mutations in the coding exons nor the adjacent intronic sites were identified in any other cell line.

Next, we performed a copy number analysis by fluorescence *in situ* hybridization (FISH) and identified heterozygous deletions of the *ETS1* locus in 2 of 7 (29%) cHL cell lines, KMH2 and SUPHD1. Expression of the ETS1 transcription factor in KMH2 is strongly down-regulated in the gene expression microarray data.[6] Interestingly, for SUPHD1, that was not included in this microarray study,

Table 1. Top 10 transcription factor motifs significantly over-represented in the group of genes hypermethylated in cHL.

Sequence motif	Transcription factor	Enrichment within genes hypermethylated exclusively in cHL4	*P**	Fold change downregulation of expression *versus* normal B-cells[14]**	Comment
V$EBF_Q6	EBF1/OLF1	1.43	5.70E-05	1.11	Down-regulated in cHL[4]
V$E12_Q6	E12 (TCF3)	1.4	3.40E-04	1.45	Impaired function in cHL[5]
V$ETS1_B	**ETS1**	**1.28**	**4.10E-04**	**7.23**	**Down-regulated in cHL (this study)**
V$E2A_Q2	E2A (TCF3)	1.17	8.30E-04	1.45	Impaired function in cHL[5]
V$MTF1_Q4	MTF1	1.71	1.20E-03	1.2	
V$OLF1_01	EBF1/OLF1	1.59	3.00E-03	1.11	Down-regulated in cHL[4]
V$HAND1E47_01	HAND1	1.17	4.40E-03	not analyzed	
V$NERF_Q2	NERF (ELF2)	1.91	5.80E-03	1.36	
V$PAX9_B	PAX9	1.3	7.20E-03	0.46	
V$MINI20_B	MINI20 (ELF2)	1.11	8.10E-03	1.36	

**Based on Fisher's exact test corrected for multiple testing (http://gather.genome.duke.edu). **Including 20 normal B-cell samples (5 x centroblasts, 5 x centrocytes, 5 x naive B cells, 5 x memory cells).*

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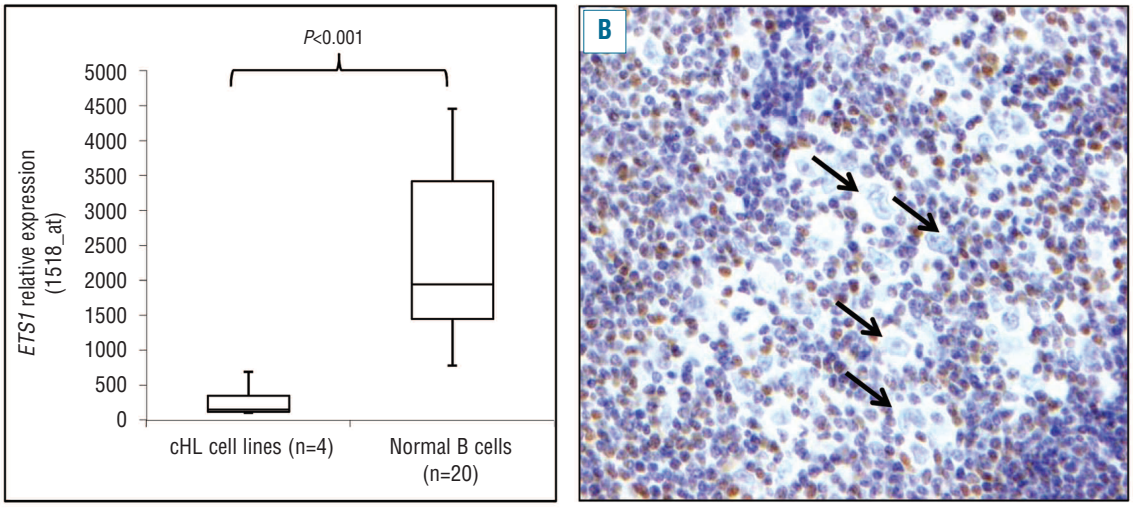

Figure 1. (A) Significant downregulation of the *ETS1* gene in cHL cell lines as compared to normal B-cell entities (5 x centroblasts, 5 x centrocytes, 5 x naive B cells, 5 x memory B cells) (Affymetrix U95 microarray tag 1518_at) prepared using (*http://www.vertex42.com/ExcelTemplates/box-whisker-plot.html*) and based on published profiles.[6] (B) cHL case with complete absence of ETS1 protein expression in HRS cells (arrows) and positively stained bystander cells.

we failed to amplify a transcript by real-time polymerase chain reaction (RT-PCR) indicating complete silencing of *ETS1* in this cell line. The size of PCR products obtained for the other 6 cell lines was as expected.

In primary cHL biopsies, 10 of 16 (63%) informative cases analyzed by FICTION showed losses of the *ETS1* locus, including one case with homozygous deletion (in 93% of HRS nuclei) and 2 cases with homozygous deletion in a sub-population of the HRS cells (in 28% and 30% of HRS nuclei, respectively). In 7 of 8 cases with *ETS1* deletion, where FICTION and immunohistochemical data were available, complete or partial loss of ETS1 protein expression was observed. Only in one of 8 cases was no reduction in ETS1 protein expression observed showing good correlation between genomic loss and *ETS1* downregulation. FISH analysis of 19 cytogenetic suspensions identified only 3 of 19 (16%) primary cases with deletion. This probably reflects the bias that arises from the difficulty of identifying HRS cells in cases not analyzed by FICTION.

Recently, non-synonymous mutations of *ETS1*[7] and copy number gains[8] probably resulting in activation of the gene were identified in diffuse large B-cell lymphomas. In contrast, the heterozygous deletions of the *ETS1* locus in HRS cells reported here can result in haploinsufficiency causing reduced expression of ETS1 protein and, consequently, of its target genes. Haploinsufficiency has already been described in hematologic malignancies; the *PAX5* transcription factor is lost in approximately 30% of cases of acute lymphoblastic leukemia.[9] It has previously been shown that ETS1 functions either as transcriptional activator or repressor, and it was reported to have oncogenic potential.[10] Furthermore, several authors reported the involvement of *ETS1* in hematopoiesis, including impaired B-cell development in mice with defective *ETS1*.[11-12]

According to these data, we propose a model in which the epigenetic downregulation of B-cell specific genes in cHL might be mediated by primary deregulation of upstream regulators of the B-cell program. We suggest *ETS1* is a player in this process and recurrent deletions and loss of expression of this transcription factor in cHL might contribute to the escape potential and survival of HRS cells.

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Key words: ETS1, transcription factor, classical Hodgkin's lymphoma, loss of B-cell identity, deletion.

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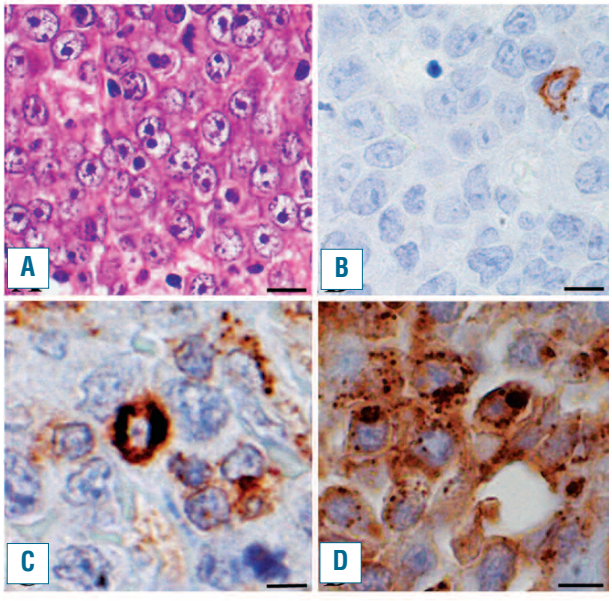
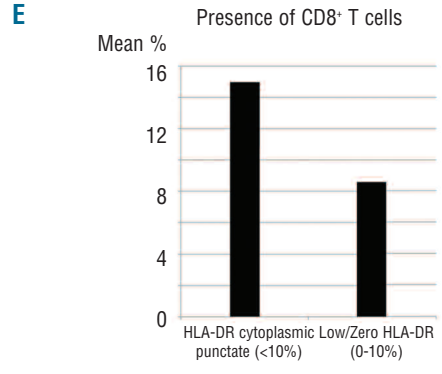
Lack and/or aberrant localization of major histocompatibility class II (MHCII) protein in plasmablastic lymphoma

Plasmablastic lymphoma (PBL) was recently reclassified as a distinct entity of mature B-cell neoplasm.[1,2] However, the diagnostic distinction of PBL from diffuse large B-cell lymphoma (DLBCL) is still a common problem due to the lack of biomarkers for PBL.[3] Recently, Montes-Moreno *et al.* published a study in Hematologica describing several PBL phenotypes that help to differentiate PBL from DLBCL.[4] A major characteristic of these PBL cases (full and variant plasmablastic phenotypes) which distinguished them from conventional DLBCL was PRDM1/Blimp1 positivity.[4] The authors further demonstrated that the rare acquisition of a partial, Blimp1 positive, plasmablastic phenotype in a minority of DLBCL cases was associated with poorer patient outcome.[4] Blimp1 and MHC class II protein expression are inversely related as normal B cells enter the terminal differentiation





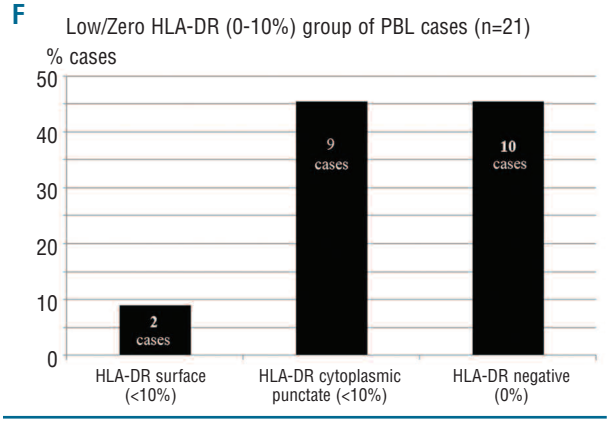


Figure 1. Hematoxylin and eosin (H&E) staining shows (A) the typical morphology of PBL. (B) CD8 staining reveals the presence of one T cell in a tumor area. (C and D) Immunohistochemical staining of PBL cases with anti-HLA-DR demonstrates the typical membrane staining in one cell (B) and the aberrant cytoplasmic punctate pattern in the absence of membrane staining (D). Bars 20 μm. (E) The "Low/Zero HLA-DR" PBL cases showed a median presence of 8.6% (±5 SD) $CD8^+$ T cells. In the one PBL case with 60% of cells strongly expressing the aberrant cytoplasmic punctate pattern in the absence of membrane staining, there were 15% $CD8^+$ T cells. (F) Within the "Low/Zero HLA-DR" group, 10 cases were completely negative. In 2 cases, a few cells (2% ±3 SD) expressed HLA-DR on the cell membrane. In 9 cases, 4% (±3 SD) of cells showed an aberrant cytoplasmic punctate pattern in the absence of membrane staining.