Chapter 6

Detection of aberrant transcription of MHC class II antigen presentation genes in chronic lymphocytic leukemia identifies HLA-DOA mRNA as a prognostic factor for survival

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Abstract
In human B cells, effective MHC class II-antigen presentation depends not only on MHC class II, but also on the Invariant chain (CD74 or Ii), HLA-DM (DM) and HLA-DO (DO), the chaperones regulating the antigen loading process of MHC class II molecules. We analyzed immediate ex vivo expression of HLA-DR (DR), Ii, DM and DO in B cell chronic lymphocytic leukemia (B-CLL). Real-time RT-PCR demonstrated a highly significant upregulation of DRA, CD74, DMB, DOA and DOB mRNA in purified malignant cells compared to B cells from healthy donors. The increased mRNA levels were not translated into enhanced protein levels but could reflect aberrant transcriptional regulation. Indeed, upregulation of DRA, DMB, DOA and DOB mRNA correlated with enhanced expression of class II transactivator (CIITA). In-depth analysis of the various CIITA transcripts demonstrated a significant increased activity of the IFN-γ-inducible promoter CIITA-PIV in B-CLL. Comparison of the aberrant mRNA levels with clinical outcome identified DOA mRNA as a prognostic indicator for survival. Multivariate analysis revealed that the prognostic value DOA mRNA was independent from the mutational status of the IGHV genes. Thus, aberrant transcription of DOA forms a novel and additional prognostic indicator for survival in B-CLL.
Enhanced MHC-II transcription in CLL

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia in adults in the Western world. A highly variable clinical course is typical for the disease is, with survival rates varying between a few months and two decades (1). B-CLL is characterized by a progressive accumulation of a malignant B cell population that fails to undergo apoptosis. Apparently, the immune system is unable to deal with this abnormal cell population. Indeed, B-CLL is characterized by striking immune incompetence in which not only the number but also the function of the B and T cells is impaired (2).

MHC class II molecules play a pivotal role in the induction and regulation of an antigen-specific immune response. MHC class II antigen presentation activates antigen-specific CD4+ T cells. The biosynthesis pathway of MHC class II is now understood in detail. MHC class II molecules bind exogenous antigens generated in the endosomal/lysosomal pathway. After synthesis, the MHC class II molecule is directed into this pathway by associating to the invariant chain (CD74 or Ii) (3). During transport to the lysosomal-like MHC class II-containing compartments (MIICs), where the majority of antigen loading occurs, CD74 is proteolytically removed, leaving only a small fragment [class II-associated invariant chain peptides (CLIP)] in the MHC class II peptide binding groove (4). Release of CLIP is facilitated by the specialized chaperone human leukocyte antigen (HLA) HLA-DM (DM), a MHC class II-like molecule. DM catalyses the natural process of peptide dissociation from MHC class II (5), thereby releasing both CLIP and other low affinity binding peptides. Consequently, DM acts as a peptide editor, favouring presentation of stable binding antigens (6-9). HLA-DO (DO), a heterodimer composed of a DOα and DOβ chain, is selectively expressed in B cells and regulates the action of DM in a pH-dependent manner. We and others reported that DO reduced MHC class II-mediated presentation of antigenic peptides in general and modulated the antigenic peptide repertoire by facilitating presentation of certain antigenic peptides, while suppressing others (10-13). DO therefore both limits and skews the class II-presented antigenic peptide repertoire in B cells. The balance between DO and DM expression thus seems to be a key factor in controlling antigen presentation in B cells, which may explain why in healthy B cells DO and DM expression are tightly regulated at various levels (14).

Transcription of MHC class II, DM and DO is regulated by a master regulator, termed the class II transactivator (CIITA) (15, 16). CIITA is transcriptionally
controlled by four distinct promoters, each transcribing a unique first exon and yielding a unique CIITA transcript (17). A physiological role for CIITA-PII is questioned as transcripts originating from this promoter are rare. The promoters I, III and IV are differentially used in different cell types and in response to inflammatory stimuli. CIITA-PI is constitutively active in myeloid dendritic cells (DCs) and CIITA-PIII constitutively in B cells, plasmacytoid DCs, monocytes and activated T cells (18). CIITA-PIV has been shown to be the promoter predominantly involved in IFN-γ-inducible CIITA expression (19, 20). In healthy B cells transcription of the MHC class II genes is tightly regulated by CIITA, but dysregulation has been observed in tumors (21-23).

The purpose of this study was to perform an overall, in depth investigation of transcription and translation of the genes involved in MHC class II antigen presentation in a small, but very well-defined B-CLL cohort and to determine whether transcriptional aberrancies occurred and if so, whether they correlated to clinical outcome.

**Materials and methods**

**Patients and healthy volunteers**

Peripheral blood samples from 21 B-CLL patients were obtained after informed consent according to the declaration of Helsinki and samples were anonymized before analysis. Patients were diagnosed and staged based on standard morphologic and immunophenotypic criteria (24). Patient characteristics are shown in Table 1. Cytogenetic data are not available because this was not routinely performed at the time of sample collection. Out of the 21 patients, 15 had not received chemotherapy at the time of sample acquisition and six patients had received prior treatment. Patients that had received treatment were equally distributed between the groups with mutated and unmutated IGHV genes. Buffy-coats from healthy donors were obtained from the Sanquin blood supply foundation (Sanquin, Amsterdam, The Netherlands).

**Purification of primary B cells**

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) and B cells were purified using anti-CD19 Dynabeads and DETACHaBEAD (Dynal, Oslo, Norway), according to the manufacturer's instructions. The cell purity and viability was >99% as
determined by FACS analysis (data not shown). Purified primary B cells were pelleted, snap frozen and stored at -80°C until further use. For purification of malignant leukaemic cells from B-CLL blood samples, mononuclear cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (Dakocytomation, Heverlee, Belgium) and phycoerythrin (PE)-conjugated anti-CD5 (Dakocytomation). The CD5+CD19+ population was purified by FACS sorting using a MoFlo Sorter (Dakocytomation), pelleted, snap frozen and stored at -80°C until further use. Purity was >99% as determined by FACS analysis (data not shown).

**Real-time PCR**

CD5+CD19+ cells were lysed in peqGOLD Trifast (PeQlab, Erlangen, Germany). GlycoBlue (Ambion, Austin, TX) was added as a carrier and total RNA was extracted according to the manufacturer’s instructions. First strand cDNA was reverse transcribed using random hexamers (pd(N)₆, Amersham Biosciences, Piscataway, NJ) and a SuperScript II, RNase H-reverse transcriptase kit (Invitrogen, Breda, The Netherlands). Gene expression was measured in the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers were selected to span exon-intron junctions to prevent amplification of genomic DNA. Primer sets (sense sequence, antisense sequence and transcript size, respectively) for the following genes were used:

- **HLA-DRA** (5'-CATGGGCTATCAAAGAAGAC-3', 5'-CTTGAGCCTCAAGCTGGC-3', 180 bp)
- **HLA-DMB** (5'-CCAGCCCAATGGAGACTG-3', 5'-CAGCCCAGGTGTCCAGTC-3', 136 bp)
- **HLA-DOA** (5'-GAGCCATACGTGCTC-3', 5'-AGTGACAGTTGGGCGTGT-3', 146 bp)
- **HLA-DOB** (5'-GGAGAAAGATGCTGAGTGCC-3', 5'-GCTCTTGAGACCTTACC-3', 133 bp)
- **CD74** (5'-CACCTGCTCAAGAATGCTG-3', 5'-CAGTTCCAGTGACTCTTC-3', 210 bp)

Total **CIITA** (5'-AACCTCAATCTGCTGAGT-3', 5'-TGTACTGGACGTCATCA-3', 191 bp)

For specific CIITA promoter transcripts, primer pairs analogous to Hornell et al. were used (25):

- **CIITA-PI** (5'-GGGAGACCTGGATTTGGCCC-3', 5'-GAAGCTCCAGGTAGCCACCTTCTA-3', 145 bp)
- **CIITA-PIII** (5'-GGGGAGGCTGAGGGCAG-3', 5'-GAAGCTCCAGGTAGCCACCTTCTA-3', 182 bp)
- **CIITA-PIV** (5'-GCCGCCAGAGCTGG-3', 5'-GAAGCTCCAGGTAGCCACCTTCTA-3', 125 bp)

As endogenous control, primers specific for human 18S rRNA were used (5'-CGGCTACCCATCAGAAGA-3', 5'-GCTGGAATTACCGCGGCT-3', 187 bp). Product specificity of each primer set was verified by agarose gel electrophoresis and by dissociation curve analysis. PCR products were further confirmed by sequence analysis. Transcript levels and relative gene expression of mRNA were determined
as described by Pfaffl (26). All results were normalized for starting template with respect to the internal control, and were expressed relative to the expression levels found in one of the CD19+ B cells from a healthy donor.

**SDS-PAGE and Western blot analyses**
The DRα-specific monoclonal antibody (mAb) 1B5 (27), DMα-specific mAb 5C1 (28), the previously described DOβ polyclonal Ab (10) and the anti-actin mAb Ab-1 (Oncogene Research Products, Boston, MA) were used for Western blot analyses. Cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA and protease inhibitors] for 30 minutes at 4°C and centrifuged for 30 minutes at 10,000 x g at 4°C. The protein content of the cell lysates was quantified using the BCA protein assay (Pierce, Rockford, IL). For Western blot analysis, equal amounts of proteins were boiled for 5 minutes in reducing Laemmli sample buffer and separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, USA) in 25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.3. Membranes were blocked in 5% skimmed milk in phosphate-buffered saline (PBS). Ab binding (in 5% skimmed milk in PBS) was detected by incubation with secondary horseradish peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit immunoglobulin Abs (Dakocytomation), followed by enhanced chemiluminescence detection (Amersham). Washes were performed using 0,1% Triton X-100 in PBS. For semi-quantitative analysis of the Western blots, subsaturated autoradiograms were scanned and the signals analyzed using TINA 2.09 software (Raytest, Staubenhardt, Germany). The relative expression levels were correlated to the cellular actin levels, measured as a reference in each sample on the blots.

**Determination of mutation status**
The mutational status of the immunoglobulin heavy chain variable (IGHV) genes of 20 patients was determined as described (29). Patient 16 had no material available for IGHV analysis. In brief, IGHV transcripts were amplified using a mixture of forward primers located in the FR1 regions of the IGHV gene families IGHV1 to IGHV6 or alternatively in the FR3 region of IGHV1 to IGHV6 in combination with one of the FAM-labelled reverse primers located in the Cμ, Cδ, Ca or Cy regions.
Less than or equal to 2% difference from the most homologous germ line gene was considered to be mutated.

**Statistical methods**

Statistical analyses were conducted with the SPSS 15.0 software program. Differences between groups were analyzed with the 2-sided Mann-Whitney \( U \) test. For correlations, the Spearman non-parametric correlation test was used. Multivariate analysis was performed using the Cox's proportional-hazards model. \( P < 0.05 \) were considered statistically significant.

Survival time after sampling was measured from the time of sampling until death, or until the end of follow-up. Survival curves were constructed using the Kaplan-Meier method. Differences between curves were analysed using the Log-rank test. The optimal cut-off value for DOA transcription levels in this patient cohort was determined using two methods: (i) the Log-rank test, by testing the prognostic value for each possible cut-off point (3.1, 3.2, 3.3 etc); and (ii) Cox regression analysis, including all cut-off points as continuous variables. Both methods gave identical results.

**Table 1.** Characteristics of the B-CLL patients included in our analyses.

<table>
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<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Months from diagnosis</th>
<th>Treatment</th>
<th>leucocyte count (x10^9/l)</th>
<th>Stage</th>
<th>Mutation status</th>
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All variables indicated were assessed at time of sampling.

Chlo, chlorambucil; Pred, prednisone. Rai and Binet refer to current standard clinical staging systems. MUT indicates mutated IGHV genes and UM unmutated and ND indicates not determined.
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Results

Transcriptional upregulation of the MHC class II components in B-CLL

To avoid patient selection biases, PBMCs were collected from a random cohort of 21 B-CLL patients, either receiving therapy or not (Table I). In purified CD5^+CD19^+, malignant B-CLL cells and mature, resting CD19^+ B cells from healthy donors, we analyzed immediate \textit{ex vivo} gene expression of DRA, CD74, DMB and both chains of the DO molecule, as unlike the DR and DM chains (16), DOA and DOB are not completely co-regulated (15, 30). Gene expression in eight samples of six independent healthy donors showed little variation in expression of DRA (0.67 (mean)±[0.37] [SD]), CD74 (0.58±[0.27]) DMB (0.79±[0.44]), DOA (0.84±[0.36]) and DOB (1.02±[0.42]) (Fig. 1), demonstrating that HLA-DR, CD74 and both class II chaperones are tightly regulated in B cells from healthy volunteers. Transcription of HLA-DR, CD74, DM and DO was markedly elevated in the malignant B-CLL cells. Overall, the patients showed a highly significant upregulation of the mRNA levels of DRA (1.49±[0.83]; \( p < 0.022 \)), CD74 (1.11±[0.39]; \( p = 0.001 \)), DMB (1.81±[0.72]; \( p = 0.001 \)), DOA (3.60±[1.47]; \( p < 0.001 \)) and DOB (4.57±[2.64]; \( p < 0.001 \)) (Fig. 1).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Transcriptional upregulation of DRA, DMB, CD74, DOA and DOB in B-CLL. Comparison of relative mRNA levels between CD19^+ B cells from healthy volunteers (= CTRL) and the CD5^+CD19^+ cells from B-CLL patients. Results are normalized to the internal control 18S rRNA and expressed relative to the value of CD19^+ B cells from a healthy volunteer.}
\end{figure}
**Downmodulation of DR, DM and DO protein expression in B-CLL**

Expression of the components of the MHC class II pathway is not only regulated at the transcriptional level, but also at the post-transcriptional and translational level (14). Thus, the anomalous transcriptional upregulation of DR, DM and DO in B-CLL is not necessarily reflected at the protein level. We analyzed the total protein expression of DR, DM and DO using SDS-PAGE and semi-quantitative Western blotting. Unlike in healthy B cells, a highly variable expression pattern of DR, DM and DO was observed in the malignant B-CLL population (Fig. 2A). Subsequent semi-quantitative analysis demonstrated that in B cells obtained from healthy donors \((n=3)\), DM and DO expression was almost invariable \((1.01±[0.03] \text{ and } 1.00±[0.03] \text{ respectively})\), with minor DR variation \((1.13±[0.21])\). Surprisingly, B-CLL cells did not express more DR \((0.73±[0.04])\), DM \((0.75±[0.18])\) and DO \((0.56±[0.37])\) protein compared to control B cells (Fig. 2B), in contrast to the significant upregulation of mRNA. DM protein is even significantly lower in B-CLL patients as compared to healthy controls.

**Increased transcription of the MHC class II genes is correlated with enhanced transcription of total CIITA in B-CLL**

Since CIITA is the master regulator of MHC class II transcription, we measured total CIITA mRNA levels in B cells from healthy donors and B-CLL patients. B-CLL patients had significant more CIITA mRNA than B cells from controls \((P < 0.001)\) (Fig. 3A). The enhanced mRNA levels of DRA, DMB, DOA and DOB correlated to the CIITA mRNA levels \((R = 0.486, P = 0.026; R = 0.530, P = 0.013; R = 0.621, P = 0.003; R = 0.614, P = 0.003 \text{ respectively})\), suggesting that enhanced transcription of these MHC class II genes is mediated by enhanced CIITA expression. As expected, CD74 mRNA expression did not correlate with CIITA expression since CD74 transcription involves other factors as well (31).

**CIITA-PIV expression is increased in B-CLL**

CIITA is a complex regulated gene, with three promoters known to display distinct cell type- and cytokine-specific responses (32). Transcription initiated by each of the CIITA promoters leads to synthesis of distinct CIITA mRNAs containing alternative first exons spliced to a shared second exon. To identify which promoter(s) of CIITA was responsible for this upregulation of CIITA, we used primers specific for the individual CIITA-PI, -PIII and –PIV transcripts.
Figure 2. Variable and deviant protein expression of DR, DM and DO in B-CLL.

(A) Western blot analysis of total cell lysates of purified, malignant B-CLL cells and B cells from healthy donors immediately after isolation. A representative healthy control is shown on the right. Equal amounts of cellular proteins were analyzed (2 μg for DR, 5 μg for DM and 15 μg for DO) as demonstrated by the comparable amounts of actin in each sample. Molecular marker sizes are indicated on the left (kDa). (B) Semi-quantitative Western blot analysis of the relative DR, DM and DO expression in healthy B cells versus malignant B cells from B-CLL patients. Subsaturated autoradiograms were scanned and signals were quantified by densitometric analysis. The values for DR, DM and DO expression were correlated to actin in each sample and subsequently expressed relative to the value obtained from a healthy control.

No messenger from CIITA-PI was found in B cells or B-CLL cells (data not shown). Transcription of CIITA-PIII mRNA was observed both in B cells from healthy donors and B-CLL patients with no significant difference between these two groups (Fig. 3B, left panel). In line with a previous report, only low levels of IFN-γ-inducible CIITA-PIV mRNA were found in B cells from healthy controls (33). In B-CLL
However, the activity of the CIITA-PIV promoter was strongly and significantly enhanced ($P < 0.001$; Fig. 3B, right panel). Thus, transcription of CIITA in B-CLL is controlled by the co-ordinated activity of the B cell-lineage-specific promoter PIII and the IFN-γ-inducible promoter PIV.

**Figure 3.** Transcriptional upregulation of CIITA in B-CLL.
Comparison of relative mRNA levels between CD19$^+$ B cells from healthy volunteers (= CTRL) and the CD5$^+$CD19$^+$ cells from B-CLL patients. Results are normalized to the internal control 18S rRNA and expressed relative to the value of CD19$^+$ B cells from one healthy volunteer.

**Prognostic value of DOA mRNA expression**

Now that we uncovered transcriptional aberrancies in the MHC II genes in B-CLL, we analyzed if differences in mRNA levels for the respective MHC II components showed a correlation with clinical outcome. Variables included were DRA, DMB, CD74, DOA and DOB, with only DOA mRNA levels showing a significant difference between surviving and non-surviving patients (Fig. 4A). If patients were divided into a group with DOA mRNA levels < 3.4 and ≥ 3.4 (the threshold with the most discriminative power in this patient cohort), DOA mRNA levels ≥ 3.4 defined a subgroup of patients with an unfavourable prognosis: six out of eight patients with DOA mRNA levels ≥ 3.4 died during follow-up as compared to four out of 12 patients with DOA mRNA levels < 3.4 (Log-rank test; $P = 0.031$, Fig. 4B). When entered as continuous variables using Cox regression analysis, a similar result was obtained ($P = 0.027$).
Figure 4. DOA mRNA expression has prognostic power in B-CLL.

(A) Comparison of DOA mRNA levels between surviving and non-surviving B-CLL patients. (B) Comparison of survival time after sampling in B-CLL according to DOA mRNA expression levels and mutation status. UM, unmutated; MUT, mutated IGHV genes.

To date, the mutational status of the IGHV genes has been considered the best prognostic marker for survival in B-CLL and indeed we confirmed this in our cohort ($P = 0.001$). When DOA mRNA levels and IGHV mutational status were entered as categorical variables in the Cox’s proportional-hazards model for multivariate analysis, both indices remained independent prognostic markers. Thus, these data indicate that aberrant DOA mRNA expression is a novel and additional prognostic indicator for survival in B-CLL, with the potential to be a risk stratifier in B-CLL patients with mutated IGHV.

Discussion

A plethora of factors has been identified that may play a role in tumor immune evasion. Some of these directly affect tumor recognition by CD8$^+$ effector CTLs, such as deficiencies in components of the MHC class I antigen processing pathway (34-37). Also, in B-CLL, MHC class I has been suggested to play a role in tumor immune evasion (38). Over the last years it has become clear that the establishment of an effective CD4$^+$ T cell response via MHC class II antigen
presentation is required for both the induction and maintenance of anti-tumor CD8+ CTL responses (39). Indeed, loss of MHC class II expression has been observed in diffuse large B cell lymphomas with fewer tumor-infiltrating CD8+ T cells in MHC class II-negative tumors (40, 41).

Here, we identified transcriptional aberrancies in the genes of the MHC class II antigen processing machinery in B-CLL. In B-CLL, the DRA, CD74, DMB, DOA and DOB were transcribed at higher levels than in healthy controls. We demonstrated that the transcriptional upregulation of the MHC II genes was not translated in increased protein levels. Therefore, the observed transcriptional aberrancies in B-CLL may not result in a form of immune evasion. Instead, it is more likely to point to an aberrant regulation of transcription. Indeed, upregulation of the transcriptional master regulator CIITA correlated to transcriptional upregulation of the MHC II genes. We demonstrated that CIITA transcription in leukaemic B cells is the resultant of both PIII and PIV promoter activity. Currently, it remains unclear why CIITA-PIV is active in B-CLL. CIITA-PIV is responsive to inflammatory stimuli. It has been shown in B-CLL that several serum cytokine levels are elevated in B-CLL (42-45) and that both CD4+ and CD8+ T cells express significantly more IFN-γ and IL-4 than in healthy controls (46, 47). Thus, activation of CIITA-PIV may be the resultant of the aberrant immunological environment in B-CLL.

Correlation of the identified transcriptional aberrancies with clinical outcome showed that the level of DOA mRNA predicts patient survival in B-CLL with high DOA mRNA levels correlating with poor survival time after sampling and low DOA mRNA correlating to good survival time after sampling. The finding that mRNA levels of DRA, CD74, DMB and DOB are not predictive indicates that transcription of DOA is not fully coregulated with the transcription of the other MHC class II genes. To validate the predictive power of DOA mRNA, we are currently expanding our cohort of B-CLL patients. The identification of DOA mRNA as a prognostic marker in our patient group is remarkable with potential clinical implication.

IGHV mutational status is the best stage-independent prognostic marker for B-CLL, but IGHV gene analysis is expensive and laborious and therefore unsuitable for standard diagnostic purposes. DOA mRNA analysis would be an additional diagnostic marker and can be readily applied in routine diagnostic purposes. Since novel therapies in the form of therapeutic antibodies or purine analogues can now induce complete remission, alternative prognostic markers become increasingly important. Even more so, the early identification of asymptomatic patients likely to progress is

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of great clinical importance as these patients are eligible for early treatment protocols. Indeed, recently other prognostic markers have been identified, with only CLLU1 (48) adding prognostic value independent of IGHV mutational status, but only in patients younger than 70 years. Here we present DOA mRNA as a novel, IGHV mutational status-independent prognostic marker for clinical outcome in B-CLL in a cohort of only 21 patients. By investigating the expression of the components of the MHC class II pathway at the transcriptional and protein level in a well defined B-CLL cohort with clinical follow-up, we defined a novel factor that is predictive for clinical outcome. Validation of this marker in a larger cohort of patients will show if DOA mRNA can be used for further tailoring therapy in B-CLL patients.

Acknowledgments
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References


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