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ABSTRACT BOOK

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ABSTRACT BOOK

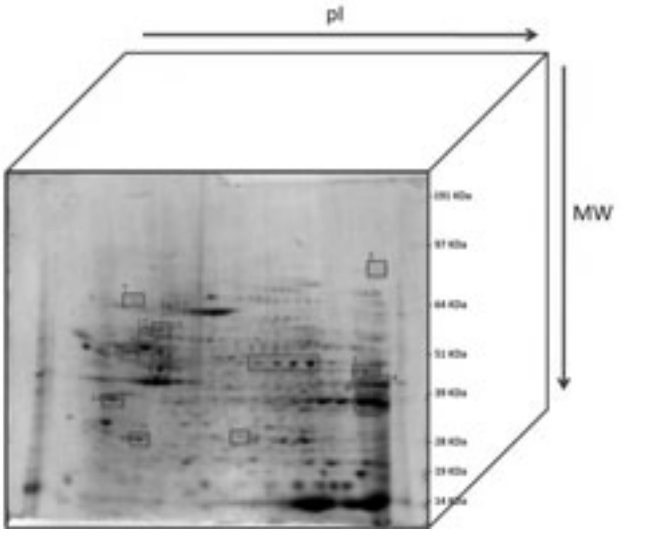

Figure 1.

tients were included in the study. Proteins extracted from leukemic cells were separated by 2-D electrophoresis (2-DE) and transferred onto membranes by electroblotting to obtain 21 2-DE proteomic maps. Each map was probed with the corresponding autologous serum by Western Blot (WB). To verify the CLL-specificity of antibodies recognition, 7 out of 21 maps obtained from CLL patients were also probed with sera collected from 7 healthy donors (HD). For identification, Ag spots in WB were aligned with proteins in 2-DE maps. The protein spots corresponding to the assigned Ag were excised from the gel, trypsin digested and analyzed by peptide mass fingerprint by MALDITOF Mass Spectroscopy (MS) with the software MASCOT. T cells isolated from the peripheral blood (PB) of 3 CLL patients with anti-ENOA antibodies were stimulated with autologous unpulsed and ENOA-pulsed dendritic cells (DC), and evaluated for their ability to secrete IFNγ through an ELISPOT assay. *Results.* Sixteen out of 21 CLL sera (76%) showed immunoreactivity against at least one Ag and produced an overall number of 45 Ag spots. By contrast, sera from HD were significantly less reactive ($p<.03$) and produced an overall number of 3 Ag spots. Eleven out of 16 (69%) reactive CLL sera recognized from 2 to 6 different Ag. All the Ag spots were characterized and consisted of 16 different proteins (Fig.1). Sera from 48% CLL patients exhibited reactivity against a protein which was identified by MS as α-Enolase (ENOA). ENOA recognition was CLL specific since none of the sera from HD showed reactivity against this protein. The IGHV mutational status was available in 20 CLL patients and 12 patients were mutated (M), while 8 patients were unmutated (UM). ENOA was recognized from sera of 7 out of 12 M patients (58%), but only from sera of 2 out of 8 UM patients (25%). The ability of ENOA to induce Ag-specific T cell responses was assessed in 3 patients. T cells isolated from the PB of CLL patients with antibody-based ENOA reactivity were stimulated with autologous ENOA-pulsed DC. The results showed that CLL-derived ENOA-pulsed DC stimulated autologous T cells to secrete IFNγ. This response was ENOA-specific because it was not induced by unpulsed DC or DC pulsed with an irrelevant protein, and also CLL-specific because IFNγ release was not induced when T cells from a HD were stimulated with autologous ENOA-pulsed DC. *Summary/Conclusions.* Our results indicate that ENOA is capable of eliciting CLL-specific humoral and cellular immune responses. Therefore, ENOA can be considered a promising biomarker and a potential target for immune-based approaches in CLL.

0650

BCR STIMULATION INDUCES A DIFFERENTIAL MICRORNA (MIR) PROFILING BETWEEN B LYMPHOCYTES DERIVED FROM CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS AND HEALTY DONORS

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Background. MicroRNAs (miRs) are small non-coding RNAs that modulate the expression of genes at the post-transcriptional level, playing a pivotal role in many physiological and pathological processes. In lymphocyte ontogenesis, an involvement of miR-181a and miR-150 in B- and T-cell development has been documented. Moreover, in chronic lymphocytic leukemia (CLL) several miRs are associated with disease pathogenesis and/or outcome. *Aims.* In order to investigate a potential role of miRs in BCR stimulation, we evaluated the expression of these small RNAs following IgM and IgD cross-linking in CLL cells, as well as in healthy B lymphocytes. *Methods.* Peripheral blood mononuclear cells isolated from 9 untreated CLL samples and 2 healthy donors were enriched in CD19+ B cells and subsequently stimulated with a F(ab')2 anti-human IgM or with a F(ab')2 anti-human IgD, at a final concentration of 10 μg/ml. Following 24 and 48 hours of incubation, total RNA was extracted from unstimulated (US) and stimulated (S) samples for miR profiling analysis, performed using the GeneChip miRNA Affymetrix arrays. An unsupervised clustering was applied to evaluate samples responsiveness to BCR ligation. To identify differentially expressed miRs between US and S cases, a t-test retaining only probesets with a p-value <0.05 and a fold change >1.5 was used. *Results.* An unsupervised approach highlighted that, after both IgM and IgD stimulation, healthy donors clustered apart from CLL cases, suggesting a differential miRs expression pattern in healthy and leukemic B lymphocytes in response to BCR engagement. Based on these findings, we performed a t-test to compare US and S cells: in agreement with the unsupervised analysis, this approach showed a homogeneous signature associated to stimulation in B cells isolated from healthy donors and allowed to identify specific sets of miRs differentially expressed following IgM and IgD ligation, respectively. In CLL, we observed the modulation of several miRs both at 24 and 48 hours of IgM cross-linking, while miR expression changes occurred exclusively at 48 hours after IgD stimulation, suggesting a delayed activation in this context. Remarkably, miRs selected in CLL S cases were different from those identified in healthy donors, confirming a distinct miR regulation in BCR signaling of these samples. *Conclusions.* Our study reveals a differential miR expression pattern following IgM and IgD ligation between CLL and healthy B lymphocytes, suggesting that distinct mechanisms regulate BCR signal transduction at the physiological and pathological level. Further investigations to combine miR and gene expression profiles obtained from the same samples are currently underway with the aim of identifying putative miR targets.

0651

CLONAL EVOLUTION IN CHRONIC LYMPHOCYTIC LEUKEMIA: ANALYSIS OF CLINICOBIOLOGIC CORRELATIONS IN 105 PATIENTS

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Background. Clonal evolution (CE) involving chromosomes 17p, 11q, 6q, and 12 was reported in 15-42% of Chronic Lymphocytic Leukemia (CLL) cases using conventional karyotyping or fluorescence *in situ* hybridization (FISH). The incidence of this phenomenon depends on the length of follow-up and on the number of probes used for interphase FISH analysis. Attention was recently devoted to 14q32 translocation involving the immunoglobulin heavy chain gene (IGH). This aberration was found in 6-19% of CLL patients at diagnosis and was associated with therapy-demanding disease and inferior outcome. The incidence of this aberration at CE is presently unknown. *Aims.* To analyze the incidence, characteristics and clinicobiological significance of CE including 14q32 translocations in CLL. *Methods.* 105 patients seen at our institution between 1995 and 2004 were analyzed sequentially by FISH with the following probes: 13q14/D13S25, 11q22/ATM, 17p13/TP53, #12-centromere and 14q32/IGH break-a-part probe. FISH analysis was performed at diagnosis or before 1st line treatment. FISH was repeated at 4-6 year intervals in patients receiving ≤1 line of treatment. In relapsed patients who started 2nd line treatment, FISH was performed sequentially before administration of the 2nd line and before each subsequent line of therapy. These 105 patients fulfilled the following criteria: diagnosis of CLL based on morphology and immunophenotyping; successful FISH analysis at diagnosis and during follow-up (cases with t(11;14)(q13;q32)/BCL1-IGH were excluded); clinical records available for review. *Results.* The median follow-up of the entire series was 73 months (range 12-180 months). CE was observed in 15/105 patients after 24-170 months (median 64). Recurring aberrations at clonal evolution were 14q32/IGH translocation in 7 patients;

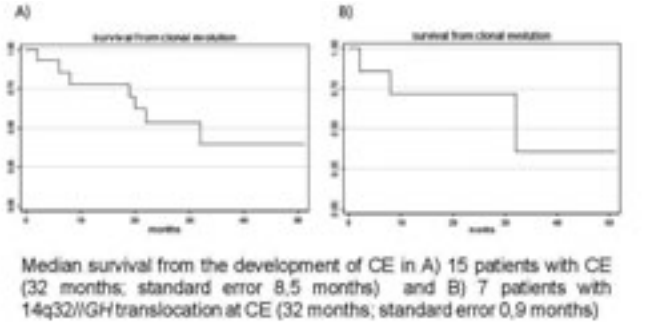

Figure 1. Survival from the development of CE.

17p- in 4 patients, 11q- in 2 patients, biallelic 13q- in 4 cases, hemizygous 13q- in 1 case, and 14q32 deletion in 1 patient. A 17p deletion was associated with 14q32/IGH rearrangement in 3/7 patients, one of whom also developed a biallelic 13q14 deletion. CE was detected in 15/58 pre-treated patients; to the contrary none of 47 untreated patients developed CE ($p<0.0001$). The 14q32/IGH rearrangement was detected after 1-4 lines of treatment (median 3 lines). In 3/7 cases with 14q32/IGH translocation BCL2 was the identified partner. In two cases the appearance of 14q32/IGH translocation was first detected in the bone marrow (BM) or in the lymph node (LN) and 13-58 months later in the peripheral blood (PB). ZAP70+ and high risk cytogenetics predicted for the occurrence of CE with borderline statistical significance ($p=0.055$ and 0.07, respectively). A shorter time to first treatment (TTT) and shorter time to chemorefractoriness (TTCR) was noted in 15 patients with CE ($p=0.033$ and 0.0046, respectively). Survival after the development of CE was 32 months (standard error 8,5). *Conclusions.* (i) 14q32/IGH translocation may represent one of the most frequent aberrations acquired during the natural history of CLL; (ii) The 14q32/IGH translocation may be detected earlier in BM or LN samples; (iii) CE including 14q32/IGH translocation occur in pre-treated patients with short TTT and TTCR; (iv) survival after CE is relatively short.

0652

ULTRA DEEP SEQUENCING OF IMMUNOGLOBULIN REARRANGEMENTS OF SEQUENTIAL SAMPLES FROM PATIENTS WITH B-CLL DEMONSTRATES CLONAL EVOLUTION

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Background and Aims. B-cell chronic lymphocytic leukaemia (CLL) is the most common leukaemia in the Western World. It is characterised by a chronic relapsing course and the development of chemotherapy resistance. There is mounting evidence that the immunoglobulin heavy chain (IgH) locus plays a central role in antigenic drive and that this may be important for disease maintenance and progression. Previous studies using next generation amplicon sequencing (NGAS) of the IgH locus have identified small sub-clones in patients with hypermutated IgH. In this study, we focussed on unmutated poor prognosis CLL and used NGAS to study clonal evolution of the IgH locus in sequential samples of the same patients. *Methods.* We amplified the clone-specific VDJ rearrangement in the immunoglobulin heavy chain using published Biomed consensus primers on sequential samples taken at diagnosis, after first treatment and at subsequent relapse on 4 CLL patients with an unmutated IgH. These products were sequenced on a 454-FLX (Roche Diagnostics). Resulting sequences were grouped using a Perl script to identify recurring reads. Reads of greater than 100 copies were analysed using Jalview and IGMT. Reads present less then 100 times were excluded from the analysis. *Results.* An average of 30000 reads were obtained for each sample. Within each sample we detected a dominant clone representing approximately 60% of all reads included in the analysis. In addition to this clone, multiple productive rearrangements were also identified when aligned to the germline using IGMT. These were present in a minority of reads. All subclones were clonally related to each other and the frequency of both dominant and additional subclones remained constant over time in the different samples despite treatment. Interestingly, one patient with an unmutated V1-69 dominant clone was found to have a hypermutated V1-69 subclone present at all three time-points. *Conclusions.* Together the data suggests that, in unmutated CLL, the leukaemic population consists of a mixture of clonally related dominant and minor IgH clones. The composition and proportion of these IgH clones remains remarkably stable over time. Further analyses of sequential samples are on-going.

0653

GENOMIC AND FUNCTIONAL ANALYSES OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELLS FOLLOWING IGD STIMULATION

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Background. Proliferation of chronic lymphocytic leukemia (CLL) cells may be influenced by antigenic stimulation and accessory signals from the microenvironment. Indeed, these factors induce different effects in distinct subgroups of patients, thus sustaining clinical heterogeneity of the disease. *Aims.* To investigate CLL responsiveness to B-cell receptor (BCR) stimulation, we evaluated the gene expression profile upon IgD cross-linking in different classes of patients, subdivided on the basis of their IGHV mutational status and clinical outcome. Microarray results were validated at the functional level using several *in vitro* assays. *Methods.* After 24 and 48 hours of incubation with a F(ab')2 anti-human IgD (10 µg/ml), unstimulated (US) and stimulated (S) CD19+ B cells isolated from untreated CLL patients underwent microarray analysis using the HGU133 Plus 2.0 Affymetrix arrays. Unsupervised clustering, t-test and Analysis of Variance (ANOVA) were performed. In addition, at 24 and 48 hours from the stimulus, antigenic expression was investigated by immunophenotypic analysis, cell cycle distribution changes were evaluated using the Acridine Orange (AO) technique, cell proliferation was measured by 3H-TdR uptake and, finally, apoptosis was analyzed by the Annexin-V and/or AO technique. *Results.* Unsupervised gene expression analysis showed that all CLL cells were responsive to stimulation, regardless of the clinico-biological features. T-test performed between US and S samples confirmed these findings and allowed to identify 290 differentially expressed genes - mostly involved in BCR signaling, cell adhesion, antigen processing and presentation, and MAPK cascade - after 24 hours of stimulation. At variance, at 48 hours, we selected 188 transcripts involved in regulation of transcription, chromatin organization, apoptosis and cell differentiation, suggesting that in CLL cells gene expression activation following IgD cross-linking occurs at later time points of stimulation. Furthermore, to assess the effects of IgD ligation in specific CLL subgroups, we used two different supervised approaches: t-test and ANOVA. Both analyses showed that the IGHV configuration and the status of the disease of the cases evaluated did not affect the responsiveness of cells to BCR engagement via sIgD. To validate the microarray results, we compared the antigen mean fluorescent intensity (MIF) ratio of US vs S cases of a set of selected transcripts encoding for B-lineage antigens involved in cell activation. This approach confirmed the downmodulation of CD79a, CD79b, CD27 and CD62L in all samples upon IgD ligation. Next, at 24 and 48 hours from the stimulus, cell cycle analysis and proliferation assay documented that IgD cross-linking induced a decrease of proliferative activity in CLL cells, irrespective of their clinico-biological characteristics; accordingly, at the same time points apoptosis increased significantly in S samples. *Conclusions.* Gene expression profile highlights that the majority of CLL are responsive to IgD stimulation, irrespective of the clinico-biological characteristics of the samples analyzed. In agreement with microarray results, *in vitro* experiments have shown a reduction of cell proliferation and a concomitant increase in the apoptotic rate of S cases, providing new insights into the mechanisms that regulate BCR engagement via sIgD in CLL.

0654

CORTACTIN EXPRESSION IS TIGHTLY CONNECTED TO B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA AGGRESSIVENESS

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Background. B-cell Chronic Lymphocytic Leukemia (B-CLL) is a disorder characterized by the accumulation of clonal CD5+ B lympho-