comparison to ZAP-70 negative group. Such a difference was observed in MFI values, however it was not statisticlly significant. The mean percentage of CD19/TNF-α cells was significantly higher, while the percentage of CD19/IL-2, CD19/IL-4 and CD19/ IFN-γ was lower in ZAP-70 positive than ZAP-70 negative patients. There was no significant difference between ZAP-70 positive and ZAP-70 negative group as far as MFI was concerned. *Conclusions.* Our findings demonstrate an association between ZAP-70 and cytokine expression in B-CLL. The more aggressive course of disease is connected with higher capability of T cells in production of cytokines responsible for disease pathogenesis. Such a connection is also observed in production of TNF-α by malignant B lymphocytes. Our results may approve the role of ZAP-70 expression by malignant cells as a good prognostic marker for B-CLL.

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0269

ACCUMULATION OF T-CELL WITH EXTREMELY SHORT TELOMERES IN T-CELL PROLYMPHOCYTIC LEUKEMIA (T-PLL)

A. Röth,[1] J. Dürig,[1] H. Himmelreich,[2] R. Siebert,[3] S. Bug,[3] U. Dührsen,[1] P. Lansdorp,[4] G. Baerlocher[2]

[1]University Hospital Essen, ESSEN, Germany; [2]University Hospital, BERN, Switzerland; [3]Institute of Human Genetics, KIEL, Germany; [4]Terry Fox Laboratory, VANCOUVER, Canada

Background. T-cell prolymphocytic leukemia (T-PLL) is a rare aggressive lymphoproliferative disease characterized by the expansion of a T-cell clone derived from immuno-competent post-thymic T-lymphocytes. Important mechanisms involved in expansion of human malignant cells are reactivation of telomerase, an enzyme complex, which is able to compensate the loss of telomere repeats by cell division, and maintenance or elongation of telomere length. *Aims.* Our aim was to investigate the role of telomeres in patients with T-PLL. *Methods.* We measured telomere length by automated multicolor flow-FISH and telomerase activity by telomeric repeat amplification protocol in subsets of peripheral blood leukocytes from 10 newly diagnosed or relapsed patients with sporadic T-PLL. *Results.* The average telomere length in the clonal T-cells of all samples analyzed was extremely short (mean±std : 1.6±0.6 kb) compared to the non-clonal T-cells (5.3±0.8 kb; p=0.012). The average telomere length for B-cells in these patients was 6.4±0.7 kb, n=5). Telomere length values of the clonal T-cells were all below the 1st percentile of telomere length values observed in T-cells from healthy aged-matched controls whereas non-clonal T-cells and B-cells fell between the 10th and 90th percentile of the normal distribution. In addition, we performed follow-up measurements of telomere length in one patient over a period of 18 months. Surprisingly, telomere length remained stably short at 1.0 kb±0.6 kb in the clonal T-cells without further telomere loss. No cell doublets indicative of fused or bridged chromosomes and telomere dysfunction were observed. Although telomerase is necessary to elongate or stabilize critical short telomeres levels of telomerase activity in the clonal T-cells were not higher than in controls. *Conclusions.* This is the first report of extremely short telomeres in clonal T-cells of patients with sporadic T-PLL. Our results are compatible with extensive proliferation of the clone. Most likely telomerase activity in T-PLL is sufficient to stably maintain extremely short telomeres and allow their clonal expansion. Current studies are aimed at exploring telomerase inhibitors to inhibit the proliferation of T-PLL cells and at the role of the very short telomeres in these cells regarding genomic instability and cytogenetic aberrations.

0270

INTRACLONAL DIVERSIFICATION OF IMMUNOGLOBULIN LIGHT CHAIN VARIABLE REGION GENES IN CHRONIC LYMPHOCYTIC LEUKEMIA

E. Kostareli,[1] K. Stamatopoulos,[2] A. Daglis,[2] C. Belessi,[3] A. Hadzidimitriou,[2] N. Stavroyianni,[2] T. Smilevska,[2] V. Tachynopoulou,[2] M. Gounari,[1] F. Marantidou,[3] N. Laoutaris,[3] A. Anagnostopoulos,[2] A. Fassas,[2] A. Kouvatsi[1]

[1]Aristotle University, Biology School, THESSALONIKI, Greece; [2]G. Papanicolaou Hospital, THESSALONIKI, Greece; [3]Nikea General Hospital, PIRAEUS, Greece

Analysis of somatic mutations in immunoglobulin heavy chain variable region (IGHV) genes in various types of B cell malignancies, including chronic lymphocytic leukemia (CLL), has demonstrated frequent intraclonal heterogeneity, indicating ongoing mutational activity. We recently showed that CLL light chain repertoire is skewed and characterized by CLL-biased features and also provided evidence for the complementary role of light chains in antigen recognition by CLL malignant cells. In the present study, we evaluated the intraclonal diversity status of IGKV/IGLV genes in 32 CLL cases; 25 cases expressed IgM/IgD, whereas 7 cases expressed IgG. IGKV-J and IGLV-J rearrangements were amplified by RT-PCR, purified, ligated into the pCRR 2.1 vector and transfected in *E. Coli*/TOP10F' cells. Sequence data were analyzed using the V-QUEST/IMGT and Clustalw/EMBL tools. Mutations observed in only one of the IGK/IGL molecular clones from the same sample were characterized as non-confirmed, whereas mutations observed more than once in the IGK/IGL molecular clones from the same sample were characterized as confirmed. The Taq DNA polymerase error rate in our laboratory is 0.052%, which may amount to 0.17 mutations/IGK or IGL clone. Overall, the cloning process was followed for 22 IGKV-J rearrangements (2/22 from lambda-expressing cases) and 10 IGLV-J rearrangements. Twelve out of 32 rearrangements (37.5%) carried IGKV/IGLV genes with greater than 98% homology to germline (*unmutated*); 5/12 *unmutated* IGKV/IGLV genes had 100% homology to germline. Information on the intraclonal variation was obtained by sequencing a minimum of 7 colonies per rearrangement. No differences were found between individual clones of 10/32 (31.2%) IGKV-J or IGLV-J rearrangements. The remaining rearrangements (22/32; 68.8%) exhibited intraclonal variation. The number of different subclones per cloning sample ranged from 3 to 5. Eight out of 32 rearrangements (25%) carried only non-confirmed mutations. Ten IGKV-J and four IGLV-J rearrangements (overall, 14/32; 43.8%) carried confirmed ongoing mutations. All nucleotide variations were single base substitutions, resulting in both silent (S) and replacement (R) mutations; nucleotide insertions or deletions were not observed. The number of nucleotide variations ranged from 1 to 7. Overall, 66 ongoing mutations (29 confirmed/ 37 non-confirmed) were observed: 24 S mutations and 42 R mutations. Twenty-nine out of 42 R mutations encoded for functionally similar amino acids. Most mutations were located in FR1/FR3/CDR1; occasional mutations were also detected in CDR2 and the IGK/L variable part of CDR3. Ongoing confirmed mutations were observed not only in *mutated* cases but also in 7/12 *unmutated* rearrangements, of which two had 100% homology. Mutations targeted A/G/C/T in a ratio of: 17/19/17/13. Transitions predominated over transversions (47 vs. 19); pyrimidines were targeted slightly more often than purines (36 vs. 30). These results indicate that IGK/IGL genes in CLL can undergo intraclonal diversification in a considerable percentage of cases and provide further support for the active contribution of light chains in antigen recognition. Mutations among subclones had specific molecular traits. Finally, intraclonal diversity did not correlate with the original mutational load, since it was observed both in CLL cases with little or no somatic mutations as in cases with considerable mutations.

0271

QUANTITATION OF MINIMAL RESIDUAL DISEASE IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA USING LNA-MODIFIED FLUORESCENTLY LABELED PROBES AND REAL-TIME PCR TECHNOLOGY

P.S. Pekova,[1] S.L. Saudkova,[2] S.L. Smolej,[2] K.T. Kozak[2]

[1]Hospital Na Homolce, PRAGUE [5], Czech Republic; [2]Dept. of Clin.Hematol., Faculty Hospital, PRAGUE, Czech Republic

Background. Patients with chronic lymphocytic leukemia (CLL) relapse even after aggressive therapies and stem cell transplantation. As the therapeutical goal today is to clear off the tumor cell burden as much as possible (by stem cell transplant or intensive chemoimmunotherapy), highly sensitive assays for minimal residual disease (MRD) evaluation and monitoring are needed. At present, many patients with not only germline IgVH sequences, but also with hypermutated IgVH genes are being treated, with the need for a sensitive and specific MRD monitoring. The original notion of MRD follow-up in CLL was based on the usage of JH-gene specific TaqMan hybridization probes. At present, due to the vast diversity of B-clonal rearrangements to be detected, the original idea has been challenged and the methodology should be modified. *Aims.* Since the hypermutation process does not restrict itself to the VH segments only and might afflict the JH segment as well, the molecular tools for the monitoring of B-CLL clonal rearrangements must be versatile enough to allow for the detection and quantitation of virtually any sequence possible. Moreover, the technique must meet the criteria for high sensitivity and specificity. We present here a novel methodology for MRD monitoring in CLL, based on LNA technology (Locked Nucleic Acids) and quantitative Real-Time PCR. *Methods.* Thirty-nine patients with the diag-

nosis of CLL were enrolled into our MRD monitoring study (16 females, 23 males, median age 59.6 yrs). 21 out of 39 individuals had unmutated IgVH genes (3 females, 18 males), 18 out of 39 patients had mutated IgVH genes (13 females, 5 males). For each patient, clone-specific primers were designed and their clonal IgVH sequences were molecularly cloned to construct the quantitation standards. In one patient, allelic inclusion has been identified (VH1-8 and VH3-30, both mutated), and for this individual, clone-specific primers and standards have been constructed for both rearrangements. To quantify the individual clonal IgVH transcripts, LNA-modified fluorescently labeled probes targeted against individual VH gene segments were employed. For any of 6 (7) IgVH families with unmutated IgVH genes, family-specific consensus LNA-modified probes were used. For those CLL cases with heavily hypermutated genes, ProbeLibraryTM was employed. For quantitation experiments, ABL was used as the control gene. *Results.* The LNA-modified probes are distinguished by a very high specificity and sensitivity (reaching to 10-8, in contrast to flow cytometry with its detection limit being 10-4). The LNA-based assays allow for precise monitoring of the residual tumor cell burden in CLL patients, especially during those periods of time, when other, less sensitive techniques fail to trace the malignant clone (during chemoimmunotherapy, after stem cell transplant). *Conclusions.* LNA-modified probes and Real-Time PCR technology represent a highly versatile, specific and extremely sensitive methodology for the monitoring of MRD in chronic lymphocytic leukemia. We strongly advocate their usage in the molecular follow-up of MRD in the setting of CLL (and possibly other B-cell malignancies with hypermutated VH gene sequences as well). CLL and related disorders - Clinical / Experimental I

0272

ADOPTIVE IMMUNOTHERAPY OF B-CELL MALIGNANCIES WITH A TRIFUNCTIONAL, BISPECIFIC ANTIBODY (ANTI-CD3 X ANTI-CD20) AND ALLOGENEIC DONOR LYMPHOCYTE TRANSFUSION

R. Buhmann,[1] M. Stanglmaier,[2] B. Simoes,[1] T. Yang,[1] P. Ruf,[2] H. Lindhofer,[2] H.J. Kolb[3]

[1]GSF, MUNICH, Germany; [2]TRION Research GmbH, Martinsried, MUNICH, Germany; [3]Klinikum Grosshadern, LMU, MUNICH, Germany

Background. CD20-directed treatment approaches turned out to be highly effective in patients with B cell non-Hodgkin's lymphoma (NHL). But although the chimeric anti-CD20 antibody rituximab induced overall response rates (ORR) of nearly 50% with median response durations of approximately 1 year in relapsed or refractory indolent lymphoma it is not curative and new immunotherapeutic treatment approaches have to be validated. *Aims.* In compassionate use, 3 patients with refractory B-cell chronic lymphocytic leukemia (B-CLL) and 3 patients with refractory high grade non-Hodgkin lymphoma (HG-NHL) were treated with the combination of the trifunctional antibody Bi20 and donor lymphocyte transfusions (DLT). *Method/Study-Design.* The Bi20 antibody is trifunctional, it binds to CD20 and CD3 and activates phagocytosis of the leukemia cell by accessory cells via the Fc part. Bi20 was applied in escalating doses from 10 µg up to 2000 µg and followed by DLT (1×10^7/kg body weight). Patient 2 and 3 received repeated courses of antibody and DLT. *Results.* In 4 out of 6 patients, we observed a prompt, but only transient clinical response. Two patients diseased from HG-NHL did not respond. In cases of B-CLL, a dose-dependent decrease of the leukemic cells was observed even within hours after antibody infusion. Moreover, enlarged lymph nodes and B-symptoms disappeared transiently. Side effects were restricted to fever, chills and bone pain that could be easily controlled. These effects peaked at a concentration of 80 µg and did not increase or even decreased at higher concentrations. The cytokine profile was characterized by a transient increase of IL-6, IL-8 and IL-10. With respect to the transaminases, only a transient and modest increase of γGT was observed. HAMAs (human anti mouse antibodies) were not detectable; their absence allowed repeated application of the trifunctional antibodies. Remarkably, graft-versus-host disease (GvHD) was not observed. Unfortunately relapse of the disease occurred in all cases. In two cases of B-CLL and one case of HG-NHL repeated application of Bi20 and T-cells induced repeated response. *Conclusion.* Bi20 can induce a prompt anti-tumor response in even extensively pretreated patients. The toxicity of treatment is tolerable. However, until now the response is of short duration and further studies are necessary to improve the outcome by e.g. optimizing the application schedule.

0273

TELOMERE LENGTH IS A PROGNOSTIC FACTOR STRONGER THAN VH-MUTATIONAL STATUS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

I. Ricca,[1] M. Ladetto,[1] A. Rocci,[1] R. Francese,[1] D. Drandi,[1] C. Lobetti Bodoni,[1] M. Compagno,[1] E. Genuardi,[1] M. Rosace,[1] M. Astolfi,[1] P. Omedè,[1] C. Marinone,[2] L. Bergui,[1] M. Boccadoro,[1] C. Tarella,[1] A. Gallamini[3]

[1]University of Turin, TORINO, Italy; [2]ASO S.Giovanni Battista, TORINO, Italy, [3]ASO S.Croce e Carle, CUNEO, Italy

Background. Telomere restriction fragments (TRF) length has a prognostic impact in B-CLL. Some studies suggest that this is a mere reflection of its association to VH-mutational status (VH-MS). However, the relative value of these two parameters has not been clearly defined, particularly in cases in which they are discordant. *Aims.* To compare, in a large population of B-CLL patients, the prognostic impact of TRF length and VH-MS, in terms of overall survival (OS), time to first treatment (TTFT) and progression free survival (PFS). PATIENTS AND *Methods.* 184 B-CLL patients have been analyzed for TRF length and VH-MS. All samples were taken before treatment start. Males were 118, females 66. Median age was 62 years (range 34-87). According to Binet staging system, 117 were stage A, 34 B and 33 C. Cytogenetics, CD38 and ZAP-70 expression were available in 80% of patients. Median follow-up was 36 months (range 6-290). Eighty-seven patients have been already treated. TRF length was evaluated by Southern blot and VH-MS by direct sequencing. The standard cut-off of 2% deviation from any germ line VH sequence was employed to define VH-MS. Survival analyses were performed using the Kaplan-Meier method. Cox multiple regression was used to analyze the independence of the following potential prognostic parameters: sex, age, Binet stage, CD38 and ZAP70 expression, cytogenetic features, VH-MS and TRF-length. *Results.* Median TRF length was 6000bp (range 1465-14837bp). There was no correlation between TRF length and patient age, sex or stage. TRF length had a major impact on prognosis with best results observed with a cut-off of 4250bp. Patients with TL<4250bp had a worse outcome than patients with TL>4250bp (median OS: 85 vs 269 months, $p<0.0001$; median TTFT: 21 vs 63 months, $p<0.0001$; median PFS: 12 vs 36 months, $p<0.0001$). VH-MS analysis was successful in 91%. Overall, discordance between VH-MS and TRF length was observed in 16% of patients. Discordance was common among VH-unmutated patients (38%) but rare among VH-mutated patients (6%). Discordant and concordant patients could not be distinguished based on VH usage or degree of homology (H) to the germline IgH sequence (i.e. H=100% vs H<100% and >99% vs H<99% and >98%). In addition they could not be distinguished based on stage, cytogenetics, CD38 and ZAP70 expression. The 24 discordant patients with VH-unmutated status and TRF length>4250bp had a clinical outcome that was significantly different from VH-unmutated patients with TRF length<4250bp (median OS: 83 vs 215 months, $p<0.05$ and median PFS: 12 vs 33 months, $p<0.05$) and similar to that of VH-mutated patients (median OS 269 months and median PFS 54 months, p=n.s.). Finally, the multivariate analysis indicated that TRF length and Binet stage were the most powerful prognostic indicators in B-CLL. *Conclusions.* Our data demonstrate that: 1) TRF length is a major prognostic indicator in B-CLL in terms of OS, TTFT and PFS; 2) when discordance exists between VH-MS and TRF length the latter better predicts outcome.

0274

HIGHLY SENSITIVE DETECTION OF MINIMAL RESIDUAL DISEASE IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA BY INTERPHASE FLUORESCENCE *IN SITU* HYBRIDIZATION ON FLOW SORTED CELLS

K. Hensen, S. Franke, H. Jongen, V. Peeters, P. Declercq, K. Magerman, A. Mewis, R. Cartuyvels, J.L. Rummens, B. Maes

Virga Jesse Hospital, HASSELT, Belgium

Background. The introduction of new therapeutic agents such as fludarabine and alemtuzumab, with or without autologous or allogeneic stem-cell transplantation, has resulted in increased complete remission rates in B-cell chronic lymphocytic leukaemia (CLL). Preliminary data have suggested that the absence of minimal residual disease (MRD) is an end point of therapy that, if achieved, translates into an improved survival. Future prospective clinical trials that aim toward achieving long-lasting complete remissions should include a test to assess MRD. However, techniques for assessing MRD in CLL show various sensitivity levels and lack standardization. *Aim.*We have developed and validated a combined method to assess MRD in CLL using fluorescence-activating