EZH2 and microRNA expression analyses in lymph nodes involved by chronic lymphocytic leukemia with proliferation centers

Ph.D Doctoral Theses

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I. INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a neoplastic disease infiltrates peripheral blood, bone marrow, spleen and lymph nodes. CLL is composed of neoplastic monomorphous, small, round-to-oval shaped atypical mature B-cells with variable scattered larger prolymphocytes and paraimmunoblasts. According to the classification of the World Health Organisation (WHO) CLL is a low-grade non-Hodgkin lymphoma origins from mature B-cells. Small lymphocytic lymphoma (SLL) is the different manifestation of the same disease without bone marrow and peripheral blood involvement. CLL is the most common leukemia in adults in western countries, approximately 7% of all non-Hodgkin lymphomas. The incidence increases with age. There is a slight male preponderance, with a male-to-female ratio of 1.7:1.

According to the infiltration pattern of neoplastic cells in the lymph nodes two histological types of CLL/SLL can be distinguished, diffuse and pseudofollicular CLL/SLL. Pseudofollicles are predominantly composed of prolymphocytes and paraimmunoblasts and considered as proliferation centres (PCs). Pseudofollicular CLL/SLL displays pseudofollicles which are confluent or larger than a 4x power field under microscope. The cells of PCs are negative for CD10, Bcl-6 and positive for Bcl-2. Among the typical small CLL cells (intervening small cell areas, IFs) scattered Ki-67 positive larger cells are present.

CLL cells show positivity for CD20, CD22, CD5, CdD9, CD79b, CD23 and CD43 and variable positivity can be found with zeta-associated protein-70 (ZAP-70). CLL is genetically heterogeneous disease. About 80% of cases have cytogenetic abnormalities detected by fluorescent in-situ hybridization (FISH). The most common alterations are 13q14.3 deletion (present in ~50% of cases), trisomy 12 (present in ~20%) and 11q22-23 deletion (present in ~25%). 17p13 and 6q21 are also common findings in CLL/SLL. MicroRNAs (miR, miRNA) miR-16-1 and -15a

are located in the deleted site of 13q, *ATM* gene is localized in 11q22-23 and *TP53* gene in 17p13. *IgHV* genes are mutated in 50-60% of the cases. In the assessment of prognosis cytogenetic abnormalities are decisive factor beside the clinical staging. Unmutated *IgHV* status, elevated ZAP-70 and CD38 expressions, del11q and del17p are poor prognostic factors. Recent studies underline the *TP53* mutational status as independent and crucial prognostic factor. CLL with expanded PCs also have worse prognosis.

During the course of CLL/SLL development of more aggressive lymphoma type occurs in 5-30% of patients. Transformation develops usually in the lymph nodes, sometimes in the bone marrow. Due to the transformation overall survival decreases dramatically. The most common transformation form is so called Richter's syndrome (RT), in which CLL/SLL transforms to diffuse large B-cell lymphoma (DLBCL). Transformation to prolymphocytic lymphoma, Hodgkin lymphoma or acute lymphocytic leukaemia rarely occurs.

Several studies confirmed the importance of PCs in progression and prognosis of the disease. Follow-up studies have been demonstrated the expansion of the number and size of PCs over time and the more aggressive behaviour of CLL/SLL with PCs is also known. With Ki-67 antibody higher proliferation can be found in the PCs and higher expressions of c-Myc and cyclin D2 are observed in PCs.

Previous study of our lab has demonstrated that cytogenetic changes accumulate in the cells of PCs. CLL cases showing pseudofollicular pattern in lymph nodes transform more often to a more aggressive form.

MicroRNAs are small, non-coding RNAs having a role in transcriptional and posttranscriptional regulation of gene expression. They regulate many physiological and pathophysiological processes including carcinogenesis. Dysregulation of miRs are widely studied in lymphomagenesis and more than 20

miRs are implicated in the development or progression of CLL/SLL.

Enhancer of zeste homologue 2 (EZH2) methyltransferase is a core member of the polycomb repressor complex 2 (PRC2) and has a critical role in several biological processes via epigenetic regulation of gene transcription. EZH2 overexpression is noted in high-grade germinal centre (GC) subtype of lymphomas, including DLBCL and follicular lymphoma (FL). Activating mutations of *EZH2* are also known in FL.

The role of EZH2 in the pathomechanism of CLL/SLL is poorly defined. In a former study higher expression of EZH2 was related to Ki-67 positive larger cells of CLL/SLL and the EZH2 overexpression is in link with worse prognosis. Cases with EZH2 overexpression are associated with elevated ZAP-70 expression or presence of cytogenetic changes or unmutated *IgHV* genes. Activating mutations of *EZH2* gene have not been described in CLL/SLL. Further assessing of EZH2 functions is important in potential anti-EZH2 target therapy. EZH2 expression is also modulated by microRNAs. MiR-101 and miR-26a are negative regulators of EZH2 and expression of EZH2 is proved to be controlled by different proteins, including E2F1, c-Myc and pRB.

In summary PCs are important sites of CLL/SLL lymph nodes. The cells of PCs have unique aspect, which indicates that PCs may have a central role in the progression of the disease, however more detailed biological features of the cells of PCs are yet to be discovered. The aim of our study was to further characterize PCs of CLL/SLL to better understand the disease. To explore the role of PCs we examined molecules the dysregulation of which are believed to negatively influence the prognosis, thus, among others, the expressions of different miRs and EZH2 in cells of PCs compared to the cells of IF regions.

II. OBJECTIVES

- Comparison of the expression levels of 19 selected miRs (miR-155, -150, -29c, -26a, -181a, -21, -15a, -16, -34a, -221, -222, -223, -101, -93-5p, -650, -92, -142-3p, -142-5p, -15b), which are implicated in the development and/or progression of CLL/SLL, in PCs and IF regions of 15 lymph node samples from CLL/SLL cases showing morphologically pseudofollicular pattern.

- Analysis of the expression level of EZH2 in PCs and IF regions.

- Analysis of the expression levels of the proteins regulate EZH2 transcription, such as E2F1, c-Myc and pRB, in PCs and IF regions.

- Analysis of activating mutations of *EZH2* gene.

III. MATERIALS AND METHODS

III. 1. Histological samples

Formalin-fixed-paraffin-embedded (FFPE) lymph node samples from 15 patients diagnosed with CLL/SLL were selected for the study. All cases were diagnosed in the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University according to the current WHO Classification of lymphoid tumours. Diagnoses were based on histopathology, immunophenotype, FISH and molecular analyses. All samples were collected at the time of initial diagnosis, before treatment. Microscopically, all cases showed pseudofollicular pattern with extended PCs. The cohort consisted of 7 female and 8 male patients, the median age at diagnosis was 58,47 years. No high-grade transfomation occurred during the period of the study, but in the last follow-up period Richter transformation in 2 cases and prolymphocytic transformation in one another case developed. There was no disease related mortality documented.

III. 2. Quantitative real-time polymerase chain reaction analysis of miR expression

In this study we examined the expression of 19 selected miRs (miR155, -150, -29c, -26a, -181a, -21, -15a, -16, -34a, -221, -222, -223, -101, -93-5p, -650, -92, -142-3p, -142-5p, -15b) implicated in the development and/or progression of CLL/SLL with quantitative real-time polymerase chain reaction (qRT-PCR) using TaqMan® MicroRNA Assay (Life Technologies). For the expression analysis first approximately 5000-5000 cells were dissected from the PCs and IF regions separately. After total RNA isolation reverse transcription of miRs were perfomed. The samples were preamplified, subsequently qRT-PCR was carried out. The relative expression levels of miRs were defind by using ΔCt method.

III. 3. Immunohistochemical analysis

To examine the expression levels of EZH2 protein and its regulators, E2F1, c-Myc and pRb in PCs and IF regions immunohistochemical analysis was performed. Novolink Polymer Detection System (Leica-Novocastra) was applied to detect antigens. After digital scanning of the slides with Pannoramic 250 Flash II high-resolution scanner (3DHISTECH Ltd.) expression levels were evaluated with Pannoramic Viewer software 1.15.4 (3DHISTECH Ltd.)

III. 4. Sequence analysis of *EZH2* and *IgHV* genes

Mutational status of *IgHV* and *EZH2* (exon 16 and 18, known activating mutations) genes were analysed with direct sequencing method. Genomic DNA was isolated from the samples. After PCR amplification bidirectional Sanger sequencing were performed using BigDye 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems). The obtained sequences were aligned to the reference human genome sequence.

III. 5. Statistical analysis

All analysis was performed with the software package SSPS (Version 20.0). Graphs were generated with Graphpad (Version 5.0). The Kolmogorov-Smirnov test was used for testing the normal distribution of the ΔCT values. The two tailed Student's test was used to evaluate the difference between protein expression and miR expression in PCs and IF regions. In case of parameters, not normally distributed Mann-Whitney U-test and Kruska-Wallis test were performed. P values 0.05 or below were considered statistically significant.

IV. RESULTS

IV. 1. MicroRNA expression in PCs and IF regions

Comparing the relative expression of 19 selected miRs believed to be implicated in the development and/or progression of CLL/SLL we found that that relative expression of 4 miRs were significantly different in PCs compared to the IFs. Expression of miR-155 and miR-92a were significantly increased and expression of miR-150 and miR-26a were significantly reduced in the cells of PCs ($p=0.005$, $p=0.011$, $p=0.009$ and $p=0.018$, respectively).

IV. 2. Expression of EZH2 protein and its regulators E2F1, c-Myc and pRb in PCs and IF regions

To detect the distribution of EZH2 protein expression and its upstream mediators E2F1, c-Myc and pRb in CLL/SLL lymph nodes of 15 patients, we digitally analysed the immunostained slides and quantified the ratios of positive cells in the PCs and IF regions. The proportion of EZH2, E2F1, c-Myc and pRb positive cells were significantly higher in the PCs compared to the IF regions ($p<0.001$, $p=0.001$, $p=0.001$ and $p<0.001$, respectively). We noticed, that principally the larger cells accumulating in PCs expressed the EZH2, E2F1, c-Myc and pRb.

IV. 3. Mutational analyses of *EZH2*

To explore whether activating mutations are responsible for the overexpression of EZH2 mutation, analyses of the hot spot regions of *EZH2* gene were performed by bidirectional Sanger sequencing in 14 cases of CLL/SLL, where DNA were available for sequencing analysis. No mutations in the hotspot regions (Y646, A682 and A692) in the exons 16 and 18 were found.

IV. 4. Sequence analysis of *IgHV* gene

Genomic DNA from 14 CLL/SLL patients were available for bidirectional Sanger sequencing of *IgHV* genes. Two cases were mutated and 12 cases unmutated. In this cohort none of the mutated cases underwent high-grade transformation during the follow-up period.

IV. 5. Correlation of miRs and EZH2, E2F1, c-Myc and pRb protein expressions with *IgHV* mutational status

Evaluating potential associations between our findings and the mutational status of *IgHV* genes there was significantly decreased miR-150 expression both in PCs and IF regions in cases bearing no mutation in the *IgHV* genes ($\Delta Ct\_^{PC}miR\text{-}150_{IgHV\_NM} = 3{,}45 \pm 0.47$, $\Delta Ct\_^{PC}miR\text{-}150_{IgHV\_M} = 4.69 \pm 0{,}50$, $t=-3{,}42$, $p=0.005$ és $\Delta Ct\_^{IF}miR\text{-}150_{IgHV\_NM} = 3.98 \pm 0.48$, $\Delta Ct\_^{IF}miR\text{-}150_{IgHV\_M} = 4.95 \pm 0.37$, $t=-2.67$, $p=0{,}021$). No correlations were detected between further miRs or protein expressions with *IgHV* mutational status.

IV. 6. Correlation of miRs and EZH2, E2F1, c-Myc and pRb protein expressions with chromosomal aberrations

Comparing the results of protein and miR expressions with chromosomal aberrations there was a tendency for lower expression of miR-150 in patients with 11q deletion ($p=0.071$) and this difference proved to be significant for the IF regions separately ($\Delta Ct\_^{IF}mir\text{-}150_{del11q+} = 3.79 \pm 0.45$, $\Delta Ct\_^{IF}mir\text{-}150_{del11q-} = 4.42 \pm 0.48$, $t=2.66$, $p=0.020$). There was a significantly reduced expression of miR-26a in the PCs of cases with chromosomal aberrations compared to the cases without cytogenetic changes ($\Delta Ct\_^{PF}miR\text{-}26a_{ChA+} = -6.15 \pm 2.08$, $\Delta Ct\_^{PF}miR\text{-}26a_{ChA-} = -$

3.5±2.01; p=0.047). MiR-26a expression was also found to be significantly reduced in the PCs of cases with 17p deletion compared to cases with other chromosomal aberrations or no cytogenetic changes ($\Delta Ct\_^{PF}miR\text{-}26a_{del17p+}$ = -8.57±2.43, $\Delta Ct\_^{PF}miR\text{-}26a_{del17p-}$ = -4.96±1.99; p=0.035).

No correlations were detected between miR or protein expressions with the other chromosomal aberrations.

V. DISCUSSION

Increasing body of data support the hypothesis that proliferation centres of CLL/SLL have a pathogenic role in the cell proliferation and progression of the disease. The genetic alterations – including cytogenetic aberrations and aberrant oncoprotein expressions – accumulate in the cells of PCs. Increased levels of cell proliferation markers, including Ki-67, cyclin D1 and c-Myc can be measured in PCs. Genetic aberrations with worse prognosis accumulate in the cells of PCs. The *IgHV* gene rearrangement is also more frequent in the cells of PCs. Overall survival of the disease was proved to be shorter in cases showing pseudofollicular pattern in lymph nodes compared to the histologically typical type showing diffuse infiltration of tumour cells in the lymph nodes. In summary several lines of evidences suggest that CLL/SLL with PCs have more aggressive clinical behaviour.

To further characterize the cells of PCs 15 FFPE lymph node samples from patients diagnosed with CLL/SLL were selected in the study. To determine whether miRs which are implicated in CLL/SLL show different expression in PCs compared to IF areas we analysed expression of 19 different miR in PCs and IFs selectively. Our study provides evidences that the expression level of miR-155 and miR-92a were significantly higher in PCs. We also detected that the expression of miR-150 and miR-26a were significantly reduced in the PCs. Our results confirm the results of *Wang et al*., who have been reported higher expression of miR-155 and lower expression of miR-150 in 8 patients with CLL/SLL with PCs, moreover support the hypothesis that the cells of PCs have distinguish features compared to IF cells.
Higher expression of oncomiR-155 in hematological malignancies was demonstrated and it has a role in the proliferation and survival through its targets SOCS1 and SHIP1 and also through the regulation of BCR signalling. The higher

expression of miR-155 in PCs may dysregulate BCR pathway which may lead to the progression of CLL/SLL.

In our study, another oncomiR, miR-92a, member of miR-17-92 cluster showed also significantly higher expression in PCs. C-Myc mediated overexpression of the cluster is already proved in lymphomas. In miR-17-92 transgenic mice develop c-Myc mediated lymphoma and *Gibson et al.* demonstrated the higher expression of c-Myc in PCs of CLL. Based on these data Myc overexpression might be responsible for the increased miR-92a level in PCs. Tumour suppressor PTEN and TGFβ are important direct targets of miR17-92 cluster. Lower expression of PTEN in CLL is described in the literature. Increased expression of miR-92a in PCs possibly induces cell proliferation and decreases apoptosis via downregulation of these tumour suppressor pathways.

The expression of miR-150 is significantly reduced in the PCs. It has been shown that miR-150 exerts a tumour suppressor function and is downregulated in several haematological malignancies including chronic myeloid leukaemia. Lower miR-150 expression is supposed to lead decreased inhibition of the BCR signalling pathway and subsequent increased proliferation. Elevated expression of FOXP1, another target of miR-150 has also been demonstrated in CLL and is related to worse prognosis of the disease. Lower expression of miR-150 probably due to the decreased inhibition of AKT signalling pathway may be responsible for the increased proliferation activity, which further confirms the hypothesis, that PCs have emphasized role in the progression.

MiR-26a expression is also significantly reduced in the PCs. Mir-26a is a negative regulator of EZH2. According to the literature overexpression of EZH2 in CLL/SLL is associated with worse prognosis. We examined the EZH2 protein expression in PCs and IFs. To determine the possible mechanism underlying EZH2 upregulation beside the quantitative RT-PCR analysis of miR-26a expression we

examined the expression level of E2F1 protein which regulates the EZH2 expression and its regulators c-Myc and phosphorylated retinoblastoma (Rb). EZH2 expression is induced by c-Myc through repression of the direct target miR-26a and by activation of transcription factor E2F1. E2F1 is also regulated by Rb, inactivation this tumour suppressor by phosphorylation leads to release and subsequent increased E2F1 activity.

Using digital quantitative analysis we verified significantly higher expression of EZH2 in PCs compared to IFs. This result is in line with a previous study of *van Kemenade et al.* who showed that EZH2 expression in low-grade lymphomas including CLL/SLL is limited to large Ki-67 positive cells.

The function of EZH2 in the pathomechanism of CLL/SLL is not yet understood. Based on our finding, overexpression of EZH2 is related to the PCs enriched with larger cells, which further supports the adverse role of PCs in the progression. Albeit no recurrent mutations of EZH2 have been described in CLL/SLL to rule out the possibility of activating mutations in the background of EZH2 overexpression we analysed exon 16 and 18 of *EZH2* gene with Sanger sequencing. We detected no mutations in the known hot spots of EZH2.

All examined proteins, c-Myc, E2F1 and pRb showed significantly higher expression in the PCs of lymph nodes of CLL/SLL. These results indicate that c-Myc/E2F1, c-Myc/miR-26a and pRB/E2F1 pathways may all be responsible for EZH2 overexpression in the PCs. These protein positivities were predominantly confined to the prolymphocytes and paraimmunoblasts which strongly suggests that EZH2 and its regulators are overexpressed in the same cells.

The miR expression of PCs and IFs, and also EZH2, E2F1, c-Myc and pRb expression levels were compared to the cytogenetic aberrations and *IgHV* mutational status. *Rabello et al.* found elevated EZH2 expression in CLL patients with cytogenetic abnormalities compared to the cases with normal karyotype. In our

study we did not found such an association, however low expression of its negative regulator miR-26a in the PCs correlated with the presence of chromosomal aberrations and isolated loss of 17p. In more than 80% of cases with 17p deletion mutation of the other *TP53* allele is present which is known to be the most important independent prognostic factor of CLL/SLL.

The lower miR-150 expression in the cells of IFs was associated with 11q deletion and decreased miR-26a expression of PCs was related to the presence of chromosomal aberrations. No association between miR-26a and 17p deletion in CLL/SLL were described in the literature so far.

In summary the results of our study provide evidence that pathogenic process involved in a more aggressive CLL/SLL takes place predominantly in the PCs.

VI. CONCLUSIONS

- Increased expression of oncomiR miR-155 and miR-92a and decreased expression of tumour suppressor miR-150 and miR-26a are detected in proliferation centres compared to the interfollicular areas in lymph nodes of CLL/SLL patients.
- Overexpression of EZH2 is verified in proliferation centres of lymph nodes of CLL/SLL patients compared to the interfollicular areas, which is induced by altered regulations in c-Myc/E2F1, c-Myc/miR-26a, pRB/E2F1 signalling pathways.
- No activating mutation of *EZH2* gene can be found as the background of elevated EZH2 expression in CLL/SLL.
- There could be a potential association between the decreased expression of miR-26a and overexpression of EZH2 and poor prognostic factors including chromosomal aberrations and *IgHV* mutational status.
- The present data support the hypothesis that proliferation centres of CLL/SLL have a pathogenic role in the progression of the disease.

VII. PUBLICATION RECORD

VII. I. Articles related to the subject of the dissertation

Kinga Szurián, Irén Csala, Violetta Piurkó, Linda Deák, András Matolcsy, Lilla Reiniger (2017) Quantitative miR analysis in chronic lymphocytic leukaemia/small lymphocytic lymphoma – proliferation centres are characterized by high miR-92a and miR-155 and low miR-150 expression. Leukemia Research; doi: 10.1016/j.leukres. 2017.04.002. **IF: 2,319**

Kinga Szurián, Irén Csala, Dóra Marosvári, Hajnalka Rajnai, Katalin Dezső, Csaba Bödör, Violetta Piurkó, András Matolcsy, Lilla Reiniger (2018) EZH2 is upregulated in the proliferation centers of CLL/SLL lymph nodes. Experimental and Molecular Pathology doi:10.1016/j.yexmp. **IF: 2,566**

VII. II. Articles in different subject

Kinga Szurian, Karl Kashofer, Bernadette Liegl-Atzwanger (2017) Role of Next-Generation Sequencing as a Diagnostic Tool for the Evaluation of Bone and Soft-Tissue Tumors. Pathobiology; 84(6):323-338. doi: 10. 1159/000478662. **IF: 1,592**

Kinga Szurian, Holger Till, Eva Amerstorfer, Nicole Hinteregger, Hans-Jörg Mischinger, Bernadette Liegl-Atzwanger, Iva Brcic (2017) Rarity among benign gastric tumors: Plexiform fibromyxoma –report of two cases. World Journal of Gastroenterology; 23(31):5817-5822. doi: 10.3748/wjg.v23.i31.5817. **IF: 3,3**

Edina Bugyik, Katalin Dezső, Eszter Turányi, **Kinga Szurián**, Sándor Paku, Péter Nagy (2012) 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene induces substantial hyperplasia in fibrotic mouse liver. International Journal of Experimental Pathology, doi: 10.1111/j.1365-2613.2011.00803.x. **IF: 2,04**

Eszter Turányi, Katalin Dezső, Edina Bugyik, **Kinga Szurián**, Sándor Paku, Péter Nagy (2010) The primary mitogen (TCPOBOP)-induced hepatocyte proliferation is resistant to transforming growth factor- β-1 inhibition. Liver international; 30(10):1505-10. doi: 10.1111/j.1478-3231.2010.02324.x. **IF: 3,84**

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