The role of the aberrant somatic hypermutation and activation-induced cytidine deaminase in the pathogenesis of mediastinal large B-cell lymphoma

Doctoral theses

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I. INTRODUCTION

Mediastinal large B-cell lymphoma (MBL) is a subtype of diffuse large B-cell lymphomas (DLBL) with distinct clinical, morphological, immunological and genetic features. MBLs are localized in the anterior mediastinum with extensive infiltration of intrathoracic structures. The tumors are composed of large, immature cells with clear cytoplasm and exhibit a diffuse growth pattern. The tumor cells express B-cell specific markers such as CD19, CD20, CD22 and CD79α, but in the majority of cases the tumor cells do not express surface or cytoplasmic immunoglobulins (Ig), despite the rearranged Ig genes.

MBL is a unique lymphoma entity in many aspects. Although chromosomal imbalances described in DLBLs involving 12q and Xq, mutations of *p53* gene have also been reported, specific genetic alterations involved in the pathogenesis of MBL are unknown. Furthermore the tumor carries numerous genetic features that are not characteristic for DLBLs: different abnormalities in the expression of MHC molecules; constitutive expression the Ig associated cell surface molecule CD79α, despite of the lack of Ig expression; and the expression of a T-cell specific gene *MAL* has also been reported in MBL.

The histogenetic derivation and cellular origin of MBL is still a matter of debate. It is believed to arise from thymic, asteroid shaped medullary B-cells, which are located around the Hassal's corpuscles. According the expression of plasma cell associated antigens (PC-1, PCA-1), the lack of the surface Ig expression and the $CD19^+$, $CD20^+$, $CD21^-$ phenotype, early immunophenotypic analyses suggested that the tumor cells of MBL has reached the final stage of B-cell differentiation.

Lately, a GC (germinal center) derivation of the tumor was proposed based on the BCL-6 and CD10 expression of tumor cells, since the expression of these markers is restricted to the GC cells of the secondary lymphoid organs. Somatic mutations detected in the variable regions of *Ig* heavy chain genes and in the 5' non-coding regions of the *BCL-6* gene also supported the idea of GC origin, since the process of somatic hypermutation (SHM) affecting the genes mentioned above takes place only in cells reached the GC stage of B-cell differentiation. However, in case of MBL no intraclonal diversity can be observed, thus the ongoing, continuous mutation activity is not characteristic for this disease. Taken together, MBL may originate from activated, post-GC cells in which the somatic hypermutation can be active in an early phase of neoplastic clones, but in later phases of the disease hypermutation activity is shut down.

Genetic instability plays an important role in the development and progression of human tumors. Aberrant somatic hypermutation (ASHM) is a recently identified form of genetic instability, regarded as a malfunction of the physiological somatic hypermutational (SHM) mechanism. The aberrant activity of the SHM process affects multiple loci outside the physiological target genes, including, proto-oncogenes *c-MYC, PAX-5, RhoH* and *PIM-1*. Thus, ASHM represents a novel and powerful mechanism of malignant transformation. ASHM has been reported in about 50% of DLBLs, however ASHM in MBL has not been described.

Activation-induced cytidine deaminase (AID) plays an essential role in the process of SHM and class-switch recombination (CSR). As a result of AID activity the conversion of deoxy-citidine (dC) to deoxy-uridine (dU) occurs, leading to formation of guanine-uracil mismatches in the DNA molecule.

Depending on the type of the specific repair mechanisms activated in order to resolve these mismatches, different types of somatic point mutations or double stranded DNA brakes could take place. Although the AID expression has a crucial role in physiologic immunological processes (SHM and CSR), its constitutive expression may promote tumorigenesis. The constitutive expression of AID results in genome-wide genetic instability through mutations, illegitimate DNA recombinations, therefore leading to the development of lymphomas. Inadequate AID expression may be responsible for the above mentioned process of ASHM, where somatic mutations appear in multiple proto-oncogenes.

Constitutive AID expression has been reported in different GC B-cell non-Hodgkin lymphomas (NHLs). Most recent studies showed AID expression in the majority of GC B-cell-like and in some of activated B-cell like DLBLs on mRNA and protein level as well, however the expression of AID in MBL has not been analyzed.

II. OBJECTIVES

In the recent study we tried to provide further insights into the pathogenesis of mediastinal large B-cell lymphoma (MBL).

Our main questions were as follows:

1. To answer the question whether the aberrant somatic hypermutation (ASHM) plays a role in the pathogenesis of MBL we have analyzed the mutational status of *c-MYC*, *PAX-5* and *RhoH* genes.

2. Gene expression analysis and immunohistochemistry was performed in order to address the possible pathogenic role of the activation-induced cytidine deaminase in the development of MBL.

3. To reveal the possible correlation between the ASHM and AID expression we have compared the results of mutational and gene expression analyses.

III. MATERIALS AND METHODS

III. 1. Histological samples

Tumor biopsies from six patients with mediastinal large B-cell lymphoma were collected during standard diagnostic procedures based on histopathologic, immunophenotypic and genotypic analyses. All lymphoma samples were classified in the 1st Department of Pathology and Experimental Cancer Research, according to the World Health Organization (WHO) classification of tumors of lymphoid tissues.

We used samples from four patients with follicular lymphoma (FL), six with diffuse large B-cell lymphoma (DLBL), and one with anaplastic large B-cell lymphoma (ALCL) as controls. In addition two reactive lymph nodes were selected. These cases were also classified according the WHO criteria.

III. 2. Cell lines

The cell line MedB-1 was used as the experimental model for mediastinal lymphoma. Its pheno- and genotypic features are similar to that of described in MBLs. Cell lines Ramos, BL-41, HT58, CEM, Jurkat and K562 were used as controls. The culturing of different cell lines was carried out according the standard procedures.

III. 3. Experiments on DNA level

During the analysis of aberrant somatic hypermutation (ASHM) genomic DNAs were isolated from 6 MBL, 1 ALCL samples and from cell lines MedB-1 and HT58. To analyze their mutational status, *c-MYC, PAX-5* and *RhoH* genes were PCR amplified and sequenced using capillary

electrophoresis. The obtained sequences were compared to the corresponding germline gene sequences available in the NCBI (National Center for Biotechnology Information) GenBank database.

III. 4. Experiments on RNA level

In order to determine the expression level of activation-induced cytidine deaminase (AID) mRNA total RNAs were isolated from 6 MBL, 4 FL, 5 DLBL, 5 normal peripheral blood samples and from MedB-1, Ramos, BL-41, HT58, CEM, Jurkat and K562 cell lines. Germinal center (GC) cells obtained by laser microdissection from 2 reactive lymph nodes were also subjects of RNA isolation. The expression level of AID was determined after reverse transcription by quantitative real-time PCR assay (Q-RT-PCR) using the $\Delta\Delta C_T$ method.

III. 5. Experiments on protein level

Paraffin embedded tissue sections for immunohistochemistry were available for four cases out of the six samples investigated on DNA and RNA level. To support our results on protein level, we carried out immunohistochemistry on four archived MBL samples and on the MedB-1 cell line. One reactive lymph node and the Jurkat cell line were used as controls. Immunohistochemistry was carried out using biotin-free conjugated polymer system. Images were taken using digital microscopy.

IV. RESULTS

IV. 1. Mutational analysis of *c-MYC*, *PAX-5* és *RhoH* genes

Six cases of MBL and the cell line MedB-1 were subjected to mutational analysis on specific regions of *c-MYC*, *PAX-5* és *RhoH* genes. These cases carried a total of 28 point mutations detected in the three investigated genes. Twenty six of these sequence variants were single nucleotide substitutions and 2 were insertions, while deletions were not found. All the six cases of MBL displayed somatic mutations in at least one of the analyzed genes. Three cases (cases 2, 4 and 5) harbored somatic mutations in one, and three cases (cases 1, 3 and 6) displayed mutations in two of the three analyzed genes. The mediastinal lymphoma cell line MedB-1 carried mutations in two genes. The mutations detected in *c-MYC* exon 1 and *RhoH* were all single nucleotide substitutions.

From a functional aspect, all mutations detected in *PAX-5* and *RhoH* genes affected non-coding regulatory regions, while some of the mutations observed in the coding sequences of the *c-MYC* gene were missense mutations. The deeper analysis of the mutational pattern showed an equal number of transitions and transversions, an almost equal ratio of affected A+T and G+C pairs, and a slightly lower involvement of the mutational hot-spot RGYW.

IV. 2. Analysis of AID mRNA expression

AID mRNA was detected in all the analyzed MBL cases. Compared to the control levels, 50- to 365-fold expression levels were detected in these cases. The AID mRNA was expressed in MBLs at comparable levels with FL (102x – 629x) and DLBL (10x – 567x) samples. The expression level of the MedB-1 cell line was also in this range, 122-fold compared to the control.

Compared to the AID expression levels of other cell lines this value was the highest one.

As expected, high levels of AID mRNA were detected in germinal center cells (1000x, 1200x), which were used as positive controls; while the expression levels of AID negative control PB samples were barely detectable (0,28x-1,71x).

Comparing the results of the mutational analysis and the expression level of AID mRNA we have not found any correlation between the number of mutations and the degree of AID expression levels. Anyway, the low number of mutations per case (2-6) and the range of the AID expression levels would not allow us to draw a definite conclusion regarding this issue.

IV. 3. Detection of AID protein

Based on the result of immunohistochemistry AID protein expression was detected in all of the four investigated cases. The majority of tumor cells showed a strong AID expression, predominantly in the cytoplasm. The AID protein was also detectable in the MedB-1 cell line, where the cells showed massive cytoplasmic and dot-like nuclear staining. As expected, the reactive lymph node showed AID positivity mainly in the region of the germinal centers, while the cell line Jurkat, used as negative control showed no AID expression at all.

V. DISCUSSION

In the present study we analyzed the mutations targeting *c-MYC, PAX-5,* and *RhoH* genes and the expression of AID mRNA in MBLs. Our results demonstrate frequent mutations of these proto-oncogenes and expression of AID mRNA, suggesting that ASHM and constitutive expression of AID may contribute to the pathogenesis of MBL.

The molecular profile of ASHM found in MBLs shares several features of that reported in DLBLs. The mutation frequency of *c-MYC, PAX-5* and *RhoH* was in the same range as observed in DLBLs, the mutations were distributed both in coding and non-coding regulatory sequences, they were mostly substitutions, and less frequently insertions or deletions. In contrast to normal somatic mutations, the number of C+G and A+T substitutions were nearly equal, the number of transitions and transversions were not different, and base pair exchanges rarely affected the RGYW motif. However, due to the limited number of cases these discrepancies might not be enough to prove statistical significance.

Physiologically, AID expression is restricted only to B cells during their maturation in GCs, while the constitutive expression of AID is associated with different GC-derived B-cell NHLs. To compare the AID expression levels of MBLs, we measured the AID mRNA expression in DLBLs and FLs as well. The AID mRNA was expressed in all MBLs and also in the MedB-1 cell line, at comparable levels with the FL and DLBL samples, but they did not reach the expression level detected in normal GC cells. Although somatic mutations and also AID expression were detected in all investigated cases, we could not observe any significant correlation between the degree of AID expression and the number of mutations per case. Besides the limited number of cases, many technical reasons could serve as explanation for this issue.

Although, a strong correlation has been reported between the expression of AID on transcriptional and protein level, we also wanted to know whether mRNA levels reflect the production of the AID protein. In all investigated tissue samples and cell lines strong AID protein expression was detected, which supported our observations made on mRNA level. Our findings about the predominantly cytoplasmic localization of AID are in line with previous studies. Considering the nuclear function of AID, these observations suggest that AID is stored in the cytoplasm and it is relocated into the nucleus during a strictly regulated process, to carry out its function.

Under physiological conditions IL-4 and CD40L co-stimulation is needed for AID expression. Effectors, such as STAT6 and NFκB play an important role during the signal transduction process. The reasons for constitutive AID expression reported in different lymphomas are not fully understood. It is possible, that constitutive AID expression in MBL is related to specific signaling pathways characteristic for this disease. Transcriptional analysis of MBL demonstrated increased expression of several *IL-4/IL-13* dependent (*CD23, NF-IL3, FIG1/IL-4I1*) genes, and some effectors of the IL-4 pathway, suggesting constitutive activation of the *IL-4/IL-13* pathways in this lymphoma.

The AID expression of MBL may reflect the GC origin of the tumor cells as it has been suggested previously by the CD10, BCL-6 and MUM1/IRF4 expression and somatic mutation of IgV_H and *BCL-6* genes of the tumor cells. Several authors proposed that the normal counterparts of the MBL tumor cells are the thymic B-cells with asteroid morphology located in the medulla of the thymus. Theories about the thymic and the germinal center origin came in harmony by a recent study in which asteroid B-cells expressing AID on mRNA and protein level as well were described in the thymic medulla. According to

these results, a GC-like reaction might take place also in the thymic asteroid B-cell, thus these cells really might correspond to the normal counterparts of the transformed MBL cells.

Although the molecular basis of ASHM is largely unknown, it has been reported that ASHM as well as constitutive and ubiquitous expression of AID may be linked to lymphoma genesis by generating mistargeted mutations in different oncogenes or tumor-suppressor genes.

The role of these two phenomenons in the process of malignant transformation has already been proved in many other lymphomas. Two recent studies suggest a possible role for AID outside the immune system during hepatocarcinogenesis and normal spermatogenesis. However, for correct interpretation of these data further studies must be done.

In summary, the correlation between the presence of somatic mutations and AID expression indicates the AID generated mutagenic effect plays a role in the pathogenesis of MBL. Furthermore, the absence of specific recurrent chromosomal translocations and the heterogeneity of genetic aberrations found in MBL also support the pathogenic role, of this hypermutation-associated genetic instability in the development of MBL.

VI. CONCLUSIONS

Original findings of the dissertation are:

- Aberrant somatic hypermutation (ASHM) is active in mediastinal large B-cell lymphoma (MBL); all the six cases of MBL displayed somatic mutations in at least one of the three analyzed genes.

- High expression of activation-induced cytidine deaminase is characteristic for MBL; the expression of AID is detectable also on protein level.

- Our results indicate that ASHM and AID expression might have a pathogenic role in the development of MBL by generating various genetic abnormalities.

VII. PUBLICATION RECORD

VII. I. Publications in the subject of the dissertation

1. **Bödör Cs**, Bognár Á, Reiniger L, Szepesi Á, Tóth E, Kopper L, Matolcsy, A. (2005) Aberrant somatic hypermutation and expression of activation induced cytidine deaminase mRNA in mediastinal large B-cell lymphoma. *British Journal of Haematology*, 129: 373-376. **IF: 4,080**

2. Reiniger L, **Bödör Cs,** Bognár Á, Balogh Zs, Csomor J, Szepesi Á, Matolcsy A. (2006) Richter's and prolymphocytic transformation of chronic lymphocytic leukemia are associated with high mRNA expression of activation-induced cytidine deaminase and aberrant somatic hypermutation. *Leukemia*, 20: 1089-1095. **IF: 6,146**

VII. II. Publications in different subject

1. Bognár Á, Csernus B, **Bödör Cs,** Reiniger L, Szepesi Á, Tóth E, Kopper L, Matolcsy A. (2005) Clonal selection in the bone marrow involvement of follicular lymphoma. *Leukemia*, 19: 1656-62. **IF: 6,612**

2. Rajnai H, **Bödör Cs,** Reiniger L, Timár B, Csernus B, Szepesi Á, Csomor J, Matolcsy A. (2006) Új lehetőség a krónikus myeloproliferatív betegségek diagnosztikájában – a JAK2 mutáció kimutatása. *Orvosi Hetilap*, 45: 2175-2179.

3. **Bödör Cs,** Matolcsy A, Bernáth M. (2007) Elevated expression of Cu, Zn-SOD and Mn-SOD mRNA in inflamed dental pulp. *International Endodontic Journal*, 40: 128-132. **IF: 1,429**

4. Balogh Zs, Reniger L, Deák L, **Bödör Cs,** Csomor J, Szepesi Á, Gagyi É, Kopper L, Matolcsy A. (2007) IgVH gene mutation status and genomic imbalances in chronic lymphocytic leukemia with increased prolymphocytes (CLL/PL). *Hematol Oncol*, 25: 90-5. **IF: 1,875**

5. Gagyi É, Horváth E, **Bödör Cs,** Timár B, Matolcsy A, Pávai Z. (2007) Prognostic significance and detection of the Internal Tandem Duplication of the FLT3 gene in acute myeloid leukemia. *Romanian Journal of Morphology and Embryology*, 47: 331-337.

6. **Bödör Cs,** Schmidt O, Csernus B, Rajnai H, Szende B. (2007) DNA and RNA isolated from tissues processed by microwave accelerated apparatus MFX-800-3 are suitable for subsequent PCR and Q-RT-PCR amplification. *Pathology and Oncology Research*, 13: 149-52. **IF: 1,241**

7. **Bödör Cs,** Rajnai H, Timár B, Csomor J, Matolcsy A. (2007) BCR-ABL mRNS expressziós szintek valós-idejű kvantitatív PCR-rel történő követése krónikus myeloid leukaemiás betegek esetében. *Hematológia Transzfuziológia*, 40: 7-14.

8. Dezső K, Jelnes P, László V, Baghy K, **Bödör Cs**, Paku S, Tygstrup N, Bisgaard HC, Nagy P. (2007) Thy-1 is expressed in hepatic myofibroblasts and not oval cells in stem cell-mediated liver regeneration. *American Journal of Pathology,* DOI: 10.2353/ajpath. 2007.070273 **IF: 5,917**

VII. III. Abstracts

1. **Bödör Cs,** Bognár Á, Szepesi Á, Matolcsy A. (2004) Genetic instability caused by somatic hypermutation in primary mediastinal lymphoma. *Tissue Antigens*, 64: 390. **IF: 1,990**

2. **Bödör Cs,** Bognár Á, Reiniger L, Szepesi Á, Matolcsy A. (2005) Aberrant somatic hypermutation and expression of actvation-induced cytidine deaminase in primary mediastinal B-cell lymphoma. *Annals Oncology*, suppl 2: v144. **IF: 4,335**

3. Bognár Á, Csernus B, **Bödör Cs,** Szepesi Á, Matolcsy A. (2004) Clonal selectioni n the bone marrow involvement of follicular lymphoma. *Tissue Antigens*, 64: 390. **IF: 1,990**

4. Bognár Á, Csernus B, **Bödör Cs,** Szepesi Á, Matolcsy A. (2005) Clonal selection in the bone marrow involvement of follicular lymphoma. *Annals Oncology*, suppl 2: v149. **IF: 4,335**

VII. IV. Presentations, posters

1. **Bödör Csaba,** Bognár Ágnes, Szepesi Ágota, Matolcsy András. Szomatikus hipermutáció okozta genetikai instabilitás mediastinális nagy B-sejtes lymphomában. (előadás) – I. díj, 63. Pathologus Kongresszus, Siófok, 2004. szeptember 23-25.

2. **Bödör Csaba,** Bognár Ágnes, Szepesi Ágota, Matolcsy András. Genetic instability caused by somatic hypermutation in primary mediastinal lymphoma. (poszter), 1st International Conference on Basic and Clinical Immunogenomics, Budapest, 2004 október 3-7 .

3. **Bödör Csaba,** Bognár Ágnes, Reiniger Lilla, Szepesi Ágota, Matolcsy András. Aberrant somatic hypermutation and expression of actvation-induced cytidine deaminase in primary mediastinal B-cell lymphoma. (absztrakt), 9th International Conference on Malignant Lymphoma, Lugano, 2005. június 8-11.

4. **Bödör Csaba,** Bognár Ágnes, Reiniger Lilla, Szepesi Ágota, Matolcsy András. Aberráns szomatikus hipermutáció és aktiváció-indukált citidin deamináz mRNS expresszió mediasztinális nagy B-sejtes limfómában.- „legjobb előadás" díj. 64. Pathológus Kongresszus, Pécs, 2005. szeptember 22-24.

5. **Bödör Csaba,** Csernus Balázs, Matolcsy András. A primer mediastinalis lymphoma sejteredete és kialakulásában szerepet játszó genetikai tényezők. Onkohematológia, mint a molekuláris medicina prototípusa – A Magyar Tudomány Napjához kapcsolódó tudományos ülés, Magyar Tudomámyos Akadémia, 2005. november 21.

6. **Bödör Csaba,** Reiniger Lilla, Bognár Ágnes, Matolcsy András. The role of aberrant somatic hypermutation and AID mRNA expression in lymphoma transformation. 16th European Congress of Immunology, Paris, 2006. szeptember 6-9.

7. **Bödör Csaba.** Akut leukémiák követése real-time PCR-rel (plenáris előadás), 65.Patológus Kongresszus, Hajdúszoboszló, 2006. október 5-7.