Somatic hypermutation of IgV_H genes and aberrant somatic hypermutation in follicular lymphoma without *BCL2* gene rearrangement and expression

Ph.D Doctoral Theses

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I. INTRODUCTION

Follicular lymphoma (FL) is the most prevalent form of indolent B-cell lymphomas, accounting for up to 20-35% of all non-Hodgkin lymphomas, and is recognized as a distinct entity in the World Health Organization (WHO) Classification of lymphoid tumours. Based on the proportion of centroblasts within the malignant follicles, FL are divided into grade 1 (predominantly centrocytes), grade 2 (centrocytes and centroblasts) grade 3A (predominantly centroblasts, but centrocytes are still present) and grade 3B (solid sheets of centroblasts) categories. Approximately 85-90% of FL carry the t(14;18)(q32;q21) chromosomal translocation, juxtaposing the *BCL2* gene with the immunoglobulin heavy chain (*IgH*) gene, resulting in the constitutive expression of BCL2 protein, which induces prolonged cell survival by blocking programmed cell death. FL is considered to derive from the germinal centre (GC) B-cells, based on the immunophenotype of tumour cells, accumulation of somatic hypermutation (SHM) in their immunoglobulin (*Ig*) genes and expression of activation-induced cytidine deaminase (AID) required for SHM.

Approximately 10-15% of FL are negative for BCL2 protein, and 5% do not exhibit the t(14;18) chromosomal translocation either. Several reports suggest that FL without translocation and expression of *BCL2* gene have distinct morphological, genetic and molecular characteristics that distinguish them from the BCL2-positive FL. These BCL2-negative FL frequently are grade 3 FL, however BCL2-negative cases were also observed in lower grade groups. Furthermore, the grade 3B FL frequently harbour translocation of the *BCL6* gene, carry trisomy of chromosome 3, and gain of chromosome 18 or 18q. It is currently unclear whether this lymphoma derives from GC or post-GC B-cells.

Based on the immunophenotype ($CD10^-$, $MUM1^+$) and frequent *BCL6* gene rearrangement, it has been suggested that these BCL2-negative FL may have a "late-stage GC" or "post-GC" cell origin, and are probably more closely related to the diffuse large B-cell lymphomas (DLBCL).

Somatic hypermutation (SHM) targets primarily the immunoglobulin variable region genes in the germinal centre. This process introduces single nucleotide substitutions, with rare deletions and duplications, resulting in the production of high-affinity antibodies and allowing affinity-maturation of the humoral immune response.

Genetic instability plays an important role in the development and progression of human tumours. Aberrant somatic hypermutation (ASHM) is a recently identified form of genetic instability, regarded as a malfunction of the physiological SHM mechanism. The aberrant activity of the SHM process affects multiple loci outside the physiological target genes, including, proto-oncogenes *BCL2*, *BCL6*, *c-MYC*, *PAX-5*, *RhoH* and *PIM1*. Thus, ASHM represents a novel and powerful mechanism of malignant transformation.

SHM has been shown to malfunction in about 50% of DLBCL, in BCL2-positive-FL, in mediastinal large B-cell lymphoma, in about 20% of AIDS-related NHL, in primary central nervous system DLBCL, in primary cutaneous marginal zone B-cell lymphoma and MALT lymphoma. ASHM has also been associated with FL and CLL transformation into higher grade DLBCL. However, ASHM in BCL2-negative FL 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 breaks could take place.

In healthy B-cell development the expression of AID is strictly regulated and restricted to GC B-cells (SHM and CSR), which follows that constitutive expression of AID may contribute to NHL formation by generating genome-wide genetic instability through mutations and illegitimate DNA recombinations. This is also supported by previous findings indicating that AID is overexpressed in BCL2-positive FL, DLBCL, Burkitt lymphoma, mediastinal large B-cell lymphoma and MALT lymphoma. AID expression in BCL2-negative FL has not yet been described.

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II. OBJECTIVES

Several reports suggest that FL without translocation and expression of *BCL2* gene have distinct morphological, genetic and molecular characteristics that distinguish them from the BCL2-positive FL.

- We characterized the mutational pattern of IgV_H genes to provide further insight into the molecular pathways of lymphomagenesis and to reveal the cellular origin of FL without *BCL2* involvement.
- We analysed whether *c-MYC*, *PAX-5* and *RhoH* proto-oncogenes are differently affected by aberrant somatic hypermutation (ASHM) in BCL2-negative FL in comparison with BCL2-positive FL.
- We determined the mRNA and protein expression level of activation-induced (cytidine) deaminase (AID) in BCL2-negative FL compared to BCL2-positive FL.

III. MATERIALS AND METHODS

III. 1. Histological samples

Lymph node (LN) biopsies of 18 patients with FL were selected for this study, based on the availability of frozen tissue for molecular analyses and formalin-fixed paraffin-embedded tissue for immunophenotyping and fluorescence *in situ* hybridization (FISH) analysis. Diagnoses were based on histopathology, immunophenotype, FISH and molecular analyses, and cases were classified according to the World Health Organization (WHO) Classification of lymphoid tumours. Peripheral blood samples from 8 healthy individuals and GC cells microdissected from 5 reactive follicles were used as controls for this study.

III. 2. DNA based analyses

DNA was extracted from 18 fresh frozen lymph node samples of cases of FL and specific gene regions were amplified. To characterize the SHM activity on IgV_H genes PCR amplicons were first cloned, then the appropriate insert of plasmid DNA was sequenced, while in case of *c-MYC*, *PAX-5* and *RhoH* genes direct sequencing was used to determine the ASHM mutational activity. The sequence analysis was performed using the IMGT/V-QUEST (International imMunoGeneTics information System, http://imgt.cines.fr) and the NCBI (National Centre for Biotechnology Information) GenBank databases.

III. 3. RNA based analyses

In order to determine the expression level of activation-induced

cytidine deaminase (AID) mRNA total RNAs were isolated from fresh frozen lymph nodes of eight cases of BCL2-negative FL and seven cases of BCL2-positive FL. Germinal centre (GC) cells obtained by laser microdissection from 5 reactive lymph nodes were also subjects of RNA isolation and used as positive controls. Furthermore, RNAs of mononuclear cells from peripheral blood (PB) of eight healthy volunteers were used as negative controls. 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. 4. Protein based analyses

To support our results on protein level, we carried out immunohistochemistry on all 18 FL samples. Immunohistochemistry was carried out using biotin-free conjugated polymer system. Images were taken using digital microscopy.

IV. RESULTS

IV. 1. Sequence analysis of IgV_H genes

PCR analysis resulted amplification in 13 cases; in 5 samples (cases 3, 4, 8, 13 and 18) IgV_H gene mutations were not analysed due to unsuccessful amplification. The closest germline V_H gene, as well as the sequence homology and mutational frequency of IgV_H genes were defined. To determine whether the tumour cells of FL had been under pressure for antigen selection, we analysed the somatic mutations of all IgV_H sequences amplified. In all analysed sequences the Chang and Casali binomial distribution model revealed more R mutations in the CDRs, and in most cases fewer R mutations in the FRs than could be expected due to chance alone, with significant ($p<0.05$) clustering. The results obtained by the multinomial model suggested by Lossos *et al.* revealed statistically significant p values, except in one case. Taken together, our results indicate the presence of antigen selection in these clones.

IV. 2. Mutational analysis of *c-MYC, PAX-5* and *RhoH* genes

Eight cases of BCL2-negative FL and seven cases of BCL2-positive FL were subjected to mutational analysis. The obtained sequences in case of *c-MYC* exon 1 and exon 2, *PAX-5* and *RhoH* genes were compared to the corresponding germline gene sequences available in the NCBI GenBank database and corresponding positions were used to localize mutations. Previously reported polymorphisms and mutations appearing more than once in separate cases, thus considered as polymorphic variants, were excluded from further analyses. Totally 7 mutations were found in BCL2-positive FL and 7 mutations in BCL2-

negative FL. The mutation pattern introduced by ASHM corresponds to the specific features of the SHM process seen in physiologic targets. Mutation distribution was similar in the examined two groups: single nucleotide substitutions predominated, with only a few insertions and no deletion. There was an equal distribution of transitions (C>T, T>C, G>A) and transversions (T>A, A>T, C>G, G>C, C>A). The ratio of G+C over A+ T substitution was elevated: 5/1 for BCL2-positive FL and 3/2 in case of BCL2-negative FL. A preferential distribution within the RGYW motif (R=A/G, Y=C/T, W=A/T) was observed only in 1 out of 6 single bp substitutions in cases of BCL2-positive FL and in 3 out of 5 mutations in cases of BCL2-negative FL.

IV. 3. Analysis of AID mRNA expression

To compare the *AID* expression of BCL2-negative and positive FL, we measured the levels of expression of *AID* mRNA in 15 cases of FL (8 BCL2-negative and 7 BCL2-positive), germinal centre B-cells and peripheral blood (PB) mononuclear cells by quantitative real-time PCR assay. These results were calculated as the mean of three independent measurements using the comparative C_T method ($\Delta\Delta C_T$). The levels of *AID* mRNA were defined as a ratio of *AID* to *β-actin* expression. Mononuclear cells from peripheral blood of eight healthy volunteers were used as negative controls and as expected a low AID expression was detected. In GC cells, used as positive controls, the measured AID expression level was high. The difference between the average level of relative expression values of follicular lymphoma without and with the presence of BCL2 was statistically not significant ($p>0.05$).

IV. 4. Analysis of AID protein expression

AID protein levels were also detected in all 18 FL cases from formalin-fixed paraffin-embedded tissue samples. There was no significant difference between the protein expression values of follicular lymphoma with and without the presence of BCL2. The AID protein was predominantly localized in the cytoplasm of germinal centre B-cells. No correlation was observed between *AID* mRNA and protein expression levels. Furthermore, no correlation has been found between FL grade, the presence or absence of BCL2 protein and AID expression. Similarly, there was no correlation between AID mRNA and protein expression levels and ASHM mutations in the *c-MYC*, *PAX-5* and *RhoH* genes. However, due to the relative small number of examined cases we were not able to make major conclusions.

V. DISCUSSION

The t(14;18) translocation and BCL2 overexpression are believed to be strongly associated with the development of FL, hence, the absence of these abnormalities in B-cell neoplasms may point to a substantially different transformational pathway and a separate entity.

To characterize a possible homogenous subtype of FL in which overexpression of *BCL2* gene is not involved, we selected FL cases showing neither the t(14;18) translocation nor the expression of BCL2 protein. The GC origin of these lymphomas was supported by a follicular growth pattern, the BCL6 and CD10 (except in 2 cases) expression of the tumour cells, and by the presence of CD21 and CD23-positive follicular dendritic reticulum cells within the neoplastic follicles. Morphological analysis of these BCL2-negative FL showed two cases of grade 2 and nine cases of grade 3A FL; there were no cases of grade 3B FL. In our series, only three out of 11 BCL2-negative samples showed *BCL6* gene alterations, in contrast with previous reports, in which a significant fraction of FL without t(14;18) translocation harboured alterations of the *BCL6* gene. *BCL6* alterations were also observed in one BCL2-positive FL from the control group.

The SHM process of *Ig* genes is a characteristic feature of FL. IgV_H sequence analysis of FL lacking *BCL2* translocation and expression revealed ongoing somatic mutations generating intraclonal heterogeneity of the tumour clones. The average mutational frequency within the IgV_H region of FL cases without *BCL2* gene rearrangement and expression was 12.76% (range 2.41% to 28.62%) which is similar to the average 11.9% frequency (range 9.79% to 16.12%) found in FL harbouring the t(14;18) translocation. The pattern and distribution of these mutations were highly consistent with antigen selection as

calculated by the binomial and the multinomial distribution models. Taken together, these results indicate that cases of FL without *BCL2* gene rearrangement and expression are derived from GC B-cells, and this subtype of FL is not distinct from FL carrying t(14;18) translocation based on the mutational pattern of IgV_H.

According to a widely accepted recent hypothesis a multistep model of tumourgenesis is instrumental in the pathogenesis of FL. The initiating genetic event is the translocation of t(14;18), causing constitutive expression of the anti-apoptotic BCL2, but antigenic stimulation and signalling through the antigen receptor is required for the neoplastic transformation. Using protein microarray analysis Zha *et al.* showed that *BCL-*X_L or AKT/BAD pathways may provide an alternative anti-apoptotic signal in FL in the absence of BCL2 protein, and our study may provide evidence that GC microenvironment and antigenic selection are also instrumental in the development of BCL2-negative FL. Our data suggest that BCL2-positive and BCL2-negative FL may have different molecular alterations at the starting point of lymphomagenesis, but in both cases the immunoglobulin receptor complex may provide additional signal(s) required for malignant transformation.

SHM occurs during the centroblast stage of B-cell maturation and requires the presence of AID. AID is highly expressed in BCL2-positive FL and DLBCL, in Burkitt lymphoma, mediastinal large B-cell lymphoma and MALT lymphoma, whereas in healthy B-cell development the expression of AID is highly regulated and restricted to GC B-cells. The constitutive expression of AID may contribute to NHL formation. To analyse whether AID expression is associated with the pathogenesis of BCL2-negative FL, we measured *AID* mRNA expression in eight cases of BCL2-negative FL and compared the results

to those of seven BCL2-positive FL. AID mRNA was expressed at various levels in both BCL2-positive and negative FL, but did not reach the level detected in normal GC cells. AID expression of FL may reflect the GC origin of the tumour cells as has also been suggested previously by the presence of somatic mutations of IgV_H genes of the tumour cells. This is consistent with previous findings showing coincidence of AID expression with ongoing type of SHM of the *Ig* genes in FL. The fact that we did not find significant differences in *AID* mRNA and protein expression levels between BCL2-positive and negative FL adds further support to the concept that these two variants of FL belong to the same entity.

SHM and AID activity have also been implicated in the alterations of proto-oncogenes such as *PIM-1*, *PAX-5*, *RhoH* and *c-MYC,* which are involved in the pathogenesis of different types of B-cell lymphomas. The rates of ASHM we found in FL without *BCL2* rearrangement and expression were lower than previously reported in DLBCL, but similar to those published by others and found by us for BCL2-positive FL, suggesting that both BCL2-positive and negative FL are targeted by a low frequency of ASHM, and BCL2 expression does not influence the incidence of ASHM in FL.

VI. CONCLUSIONS

- Our findings suggest that FL may develop even without the involvement of the *BCL2* gene, although, BCL2 protein overexpression is considered to be a critical pathogenic event in the development of FL.

- Our findings demonstrate that follicular lymphomas without *BCL2* gene rearrangement and expression are associated with ongoing type of somatic hypermutation (SHM) of the IgV_H genes, low activity of aberrant somatic hypermutation and elevated activation-induced cytidine deaminase expression. These results are in concordance with the results found in the BCL2-positive follicular lymphoma cases.

- These data suggest that BCL2-positive and BCL2-negative FL may have different molecular alterations at the starting point of lymphomagenesis, but in both cases the GC microenvironment and the immunoglobulin receptor complex may provide additional signal(s) required for malignant transformation.

- The present data support the hypothesis that BCL2-positive and BCL2-negative follicular lymphomas (grades 1-3A) represent a homogenous group with different initial but several common additional molecular pathways which lead to highly similar morphological, immunophenotypic and also molecular features.

VII. PUBLICATION RECORD

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

1. Rajnai H, Bödör Cs, Balogh Zs, **Gagyi É**, Csomor J, Krenács T, Tóth E, Matolcsy A. Impact of the reactive microenvironment on the bone marrow involvement of follicular lymphoma. *Histopathology*. 2012 DOI: 10.1111/j.1365-2559.2012.04187.x **IF: 3.569**

2. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. Somatic hypermutation of IgV_H genes and aberrant somatic hypermutation in follicular lymphoma without *BCL-2* gene rearrangement and expression. *Haematologica,* 2008;93:1822-1828. **IF: 5.978**

3. Balogh Zs, Reiniger L, Deák L, Bödör Cs, Csomor J, Szepesi Á, **Gagyi É**, Kopper L, Matolcsy A. IgV_H gene mutation status and genomic imbalances in chronic lymphocytic leukemia with increased prolymphocytes (CLL/PL). *Hematological Oncology*, 2007;25: 90-95. **IF: 2.180**

VII. II. Articles in different subject

1. Castellanos KJ, **Gagyi É**, Kormos B, Valyi-Nagy K, Voros A, Shukla D, Horvath S, Slavin KV, Valyi-Nagy T. Increased Axonal Expression of Nectin-1 in Multiple Sclerosis Plaques. Neurological Sciences, 2012 DOI: 10.1007/s10072-012-1026-9. **IF: 1.22**

2. **Gagyi É**, Kormos B, Castellanos K, Valyi-Nagy K, Korneff D, LoPresti P, Woltjer R, Valyi-Nagy T. Decreased oligodendrocyte nuclear diameter in Alzheimer's disease and Lewy body dementia. Brain Pathology, 2012 DOI: 10.1111/j.1750-3639.2012.00595.x. **IF: 4.741**

3. Balogh Z, Reiniger L, Rajnai H, Csomor J, Szepesi A, Balogh A, Deák L, **Gagyi É**, Bödör C, Matolcsy A. High rate of neoplastic cells with

genetic abnormalities in proliferation centers of chronic lymphocytic leukemia. *Leuk Lymphoma,* 2011;52(6):1080-4. **IF: 2.492**

4. Dosa S, Castellanos K, Bacsa S, **Gagyi É**, Kovacs SK, Valyi-Nagy K, Shukla D, Dermody TS, Valyi-Nagy T. Chronic progressive deficits in neuron size, density and number in the trigeminal ganglia of mice latently infected with herpes simplex virus. *Brain Pathology,* 2011;21(5):583-93. **IF: 4.741**

5. **Gagyi É**, Horváth E, Bödör Cs, Timár B, Matolcsy A, Pávai Z. Prognostic significance and detection of the Internal Tandem Duplication of the FLT3 gene in acute myeloid leukemia. *Romanian Journal of Morphology and Embryology,* 2006;47:331-337.

VII. III. Book chapter

Valyi-Nagy K, Voros A, **Gagyi É**, Valyi-Nagy T. (2011) Increased resistance of vasculogenic mimicry-forming uveal melanoma cells against cytotoxic agents in three-dimensional cultures. In: Research on Melanoma - A Glimpse into Current Directions and Future Trends. Murph Mandi (Ed.), InTech, Vienna, Austria. ISBN: 978-953-307-293-7.

VII. IV. Abstracts

1. Rajnai H**,** Bödör Cs, Balogh Zs, **Gagyi É**, Csomor J, Krenács T, Tóth E, Matolcsy A**.** (2011) Impact of the microenvironment during bone marrow involvement of follicular lymphomas. Virchows Archive, 459: p.S33. **IF: 2.336**

2. Balogh Zs, Reiniger L, Rajnai H, Csomor J, Szepesi Á, Balogh A, Deák L, **Gagyi É**, Bödör Cs, Matolcsy A. (2009) High rate of genetic abnormalities of neoplastic cells in pseudofollicles of CLL. Chromosome Research, 17, suppl 1:152. **IF: 3.405**

3. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. (2008) Bcl-2 negative follicular lymphoma is associated with somatic hypermutation of the IgV_H genes

and aberrant somatic hypermutation. Journal of Hematopathology, 1: 180.

4. Balogh Zs, Reiniger L, Deák L, Bödör Cs, Csomor J, Szepesi Á, **Gagyi É**, Kopper L, Matolcsy A. (2008) IgVH gene mutation status and genomic imbalances in chronic lymphocytic leukaemia with increased prolymphocytes (CLL/PL). Journal of Hematopathology, 1:220.

5. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. (2008) Bcl-2 negative follicular lymphoma is associated with somatic hypermutation of IgV_H genes and aberrant somatic hypermutation. *Revista de Medicina si Farmacie/Orvosi és Gyógyszerészeti Szemle*, 54: 211-213.

6. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. (2008) Bcl-2 negative follicular lymphoma is associated with somatic hypermutation of the IgV_H genes and aberrant somatic hypermutation. Annals of Oncology, 19, suppl 4: 395. **IF: 4.935**

7. Balogh Zs, Reiniger L, Deák L, Bödör Cs, Csomor J, Szepesi Á, **Gagyi É**, Kopper L, Matolcsy A. (2008) IgVH gene mutation status and genomic imbalances in chronic lymphocytic leukaemia with increased prolymphocytes (CLL/PL). Annals of Oncology, 19, suppl 4: 400. **IF: 4.935**

VII. V. Presentations, posters

1. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. A bcl-2 negatív follicularis lymphomák szomatikus hipermutációs és aberráns szomatikus hipermutációs aktivitása. (presentation) 67. Pathologus Kongresszus, Keszthely, 2008 October 9-11.

2. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. Bcl-2 negative follicular lymphoma is associated with somatic hypermutation of the IgV_H genes and aberrant somatic hypermutation. (poster, LS 8) XIV. Meeting of the European Association for Haematopathology, Bordeaux, 2008 September 20-25.

3. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. Bcl-2 negative follicular lymphoma is associated with somatic hypermutation of IgV_H genes and aberrant somatic hypermutation. (presentation) The First Conference of PhD Students in Medicine and Pharmacy, Marosvásárhely, 2008 July 9-11.

4. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. Bcl-2 negative follicular lymphoma is associated with somatic hypermutation of the IgV_H genes and aberrant somatic hypermutation. (abstract) 10^{th} International Conference on Malignant Lymphoma, Lugano, 2008 June 4-7.

5. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. A bcl-2 negatív follicularis lymphomák szomatikus hypermutációs és aberráns szomatikus hypermutációs aktivitása. (presentation) Semmelweis Egyetem PhD Tudományos Napok, 2008 April 10-11.

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