Gain of chromosome region 18q21 including the *MALT1* gene is associated with the activated B-cell-like gene expression subtype and increased *BCL2* gene dosage and protein expression in diffuse large B-cell lymphoma

Judith Dierlamm,[1] Eva M. Murga Penas,[1] Stefan Bentink,[2] Swen Wessendorf,[3] Hilmar Berger,[4] Michael Hummel,[5] Wolfram Klapper,[6] Dido Lenze,[5] Andreas Rosenwald,[7] Eugenia Haralambieva,[7] German Ott,[7] Sergio B. Cogliatti,[8] Peter Möller,[9] Carsten Schwaenen,[3] Harald Stein,[5] Markus Löffler,[4] Rainer Spang,[2] Lorenz Trümper,[10] and Reiner Siebert[11] for the Deutsche Krebshilfe Network Project "Molecular Mechanisms in Malignant Lymphomas"

[1]Dept. of Oncology and Hematology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [2]Max Planck Institute for Molecular Genetics, Computational Diagnostics Group, Berlin, Germany; [3]Dept. of Internal Medicine III, Hematology and Oncology, University Hospital Ulm, Ulm, Germany; [4]Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany; [5]Institute of Pathology, Campus Benjamin Franklin, Charité-University Medicine Berlin, Berlin, Germany; [6]Dept. of Pathology, Hematology Section and Lymph Node Registry, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany; [7]Institute of Pathology, University of Würzburg, Würzburg, Germany; [8]Institute of Pathology, Kantonsspital St. Gallen, St. Gallen, Switzerland; [9]Institute of Pathology, University of Ulm, Ulm, Germany; [10]Department of Hematology, University Hospital Göttingen, Göttingen, Germany and [11]Institute of Human Genetics, University Hospital Schleswig-Holstein Campus Kiel, Kiel, Germany

ABSTRACT

Background

The aim of this study was to determine the impact of a gain of the *MALT1* gene on gene expression and clinical parameters in diffuse large B-cell lymphoma.

Design and Methods

We analyzed 116 cases of diffuse large B-cell lymphoma by fluorescence *in situ* hybridization, array-based comparative genomic hybridization, and transcriptional profiling.

Results

A gain of 18q21 including *MALT1* was detected in 44 cases (38%) and was accompanied by a gain of *BCL2* in 43 cases. All cases with a 18q21/*MALT1* gain showed BCL2 protein expression, whereas 79% in the group without a 18q21/*MALT1* gain did so ($p<0.001$). Cases with 18q21/*MALT1* gain more frequently showed an activated B-cell-like (ABC) gene expression signature (65%) than a germinal center B-cell-like (GCB) one (23%) ($p<0.001$). Ninety-eight genes including *MALT1*, *BCL2*, and some selected nuclear factor-κB target genes were differentially expressed between the two genetic groups of diffuse large B-cell lymphoma. By global testing of each chromosome, we identified 33 genes, all located on chromosome 18q, which were differentially expressed between the two genetic groups independently of the ABC/GCB status. In multivariate analysis, the 18q21/*MALT1* status represented an independent negative prognostic factor for overall survival ($p=0.03$).

Conclusions

In diffuse large B-cell lymphoma, gain of 18q21 including *MALT1* is significantly associated with differential expression of genes located on 18q, the ABC gene expression subtype, increased *BCL2* gene and protein expression and might indicate an unfavorable prognosis.

Key words: *MALT1*, *BCL2*, gene expression, prognosis, diffuse large B-cell lymphoma.

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Correspondence:
Judith Dierlamm, M.D., Ph.D., Department of Oncology and Hematology with the sections of Bone Marrow Transplantation and Pneumology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany.
E-mail: judith_dierlamm@yahoo.de

Further members of the Deutsche Krebshilfe Network Project "Molecular Mechanisms in Malignant Lymphomas" who contributed to the present work are: TFE Barth, C Becher, H-W Bernd, C Bokemeyer, AC Feller, M-L Hansmann, L Harder, D Hasenclever, K Hinz, MD Kühn, P Lichter, JI Martin-Subero, H-K Müller-Hermelink, C Pott, M Rosolowski, B Stuerzenhofecker, M Szczepanowski, H Trautmann, and H-H Wacker.

The online version of this article contains a supplementary appendix.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoma in adults, accounting for 30% to 40% of lymphoid neoplasms.[1] The diversity in clinical presentation and outcome, as well as the pathological and biological heterogeneity suggest that DLBCL comprises several disease entities that may require different therapeutic approaches.[2-4]

Recent gene expression profiling studies have identified several prognostically different subgroups of DLBCL with gene expression patterns indicative of the different stages of B-cell differentiation, termed germinal center B-cell-like (GCB), activated B-cell-like (ABC), and primary mediastinal (PM) DLBCL,[5-7] as well as activation of distinct biological pathways, especially oxidative phosphorylation, B-cell receptor/proliferation, and host response.[8,9] In addition, we recently defined the gene expression signature of Burkitt's lymphoma and showed that a small proportion of DLBCL also bears this signature.[10] Patients with a "molecular Burkitt's lymphoma signature" were younger, in a less advanced clinical stage, and a had better clinical outcome compared to patients without this signature.[10] The 5-year survival rates of patients with GCB-DLBCL, ABC-DLBCL, and PM-DLBCL are 59%, 30%, and 64%, respectively.[5-7] GCB-DLBCL is characterized by recurrent *REL* amplification, *BCL2* translocations,[6,11] and ongoing somatic hypermutation of the immunoglobulin genes.[12] In contrast, ABC-DLBCL has constitutively activated transcription factor nuclear factor (NF)-κB, which plays a critical role in the pathogenesis of this lymphoma,[13-15] and BCL2 protein overexpression in the absence of the t(14;18).[16] The mechanisms underlying BCL2 overexpression in t(14;18)-negative DLBCL are largely unknown. Since *BCL2* is a target gene for NF-κB[17] and NF-κB is constitutively expressed in ABC-DLBCL, BCL2 upregulation may be mediated through NF-κB. Another possible mechanism resulting in BCL2 protein overexpression is a gain of *BCL2*, as recently shown by Iqbal *et al.*[16]

In this scenario, the role of the *MALT1* gene, which lies close to *BCL2* on chromosomal band 18q21 and has an important impact on NF-κB activation, was not known and was, therefore, explored in the present study.

Design and Methods

Patients

This study is part of the German network project *Molecular Mechanisms of Malignant Lymphomas*, for which ethic approval was obtained. Mature aggressive B-cell lymphomas with more than 70% of tumor cells were analyzed by a comprehensive approach including gene expression profiling, array-based comparative genomic hybridization (array-CGH), and interphase fluorescence *in situ* hybridization (FISH).[10] The diagnoses were established by seven expert hematopathologists, who evaluated morphology and immunohistochemistry in a centralized panel review as previously described.[10] The intensity of BCL2 expression by the tumor cells was determined in relation to that of BCL2-positive reactive T-cells. Only lymphomas with an intensity of BCL2 expression similar to or stronger than that of reactive T cells and with more than 25% of BCL2-positive tumor cells were scored as BCL2-positive.

We included all cases analyzed in the above mentioned network project until December 2005 which fulfilled the following criteria: (i) a histopathologic diagnosis of DLBCL according to the WHO classification, (ii) lack of a molecular Burkitt's lymphoma signature, (iii) samples not from patients with relapse, human immunodeficiency virus-infection, or PM-DLBCL, (iv) interphase FISH evaluable for imbalances of the *BCL2* and *MALT1* loci.

From 142 DLBCL cases with available interphase FISH results, 10 cases were excluded because they carried a molecular Burkitt's lymphoma signature and 16 further cases were excluded because relapse of the lymphoma, a human immunodeficiency virus-infection, or a PM-DLBCL was diagnosed. None of the ten DLBCL with a molecular Burkitt's lymphoma signature showed a gain of *MALT1* or *BCL2*. Thus, a total of 116 cases entered the study (Table 1). Clinical and survival data were available for 81 of these patients (Table 2). The median follow-up time of these patients was 5 years (range, 0 to 209 months).

Fluorescence in situ hybridization

Interphase FISH was performed on 5 µm thick frozen tissue sections according to standard methods. Two hundred interphase nuclei were analyzed in each case by at least two independent investigators. Gene amplification was defined by the presence of a cloud-like signal accumulation or more than five individual signals in the interphase cells. A gain of the respective gene was defined by the presence of three to five hybridization signals per interphase nucleus occurring in more than 5% of nuclei. In cases with a gain of *MALT1* or *BCL2*, as shown by interphase FISH and a balanced array-CGH status for these genes, further FISH analyses were evaluated for imbalances of the *BCL6*, *MYC*, and *IGH* loci to differentiate polysomies of 18q21 from polyploid karyotypes without a real 18q21 gain. The *MALT1* and the *BCL2* loci were analyzed using dual color FISH with probe LSI® MALT1 Dual Color, Break Apart Rearrangement Probe and probe LSI® IGH/BCL2 Dual Color, Dual Fusion Translocation Probe, respectively (both from Abbott Vysis, Downers Grove, IL, USA). In addition, as described by Hummel *et al.*,[10] breakpoints within the *MYC*, *BCL6*, and *IGH* genes were analyzed with the LSI® MYC, LSI® BCL6, and LSI® IGH break apart assays (all from Abbott Vysis) and *MYC* partners with the LSI® IGH/MYC (Abbott Vysis) and home-brewed IGK-MYC and IGL-MYC double-color, double-fusion assays.[10]

Array-CGH

Array-CGH was performed as previously described.[10,18,19] A 2.8k array CGH chip containing 2799

fragments was applied. The previously published *GoldenPath* clone set[20] served as the backbone for the set up of the chip. In total, 1500 DNA clones at intervals of approximately 2 Mb were selected. This compilation was enriched by 600 DNA fragments, which represent critical regions in B-cell neoplasms as well as 699 additional DNA fragments containing proto-oncogenes or tumor suppressor genes. DNA preparations of all BAC and PAC clones and subsequent degenerate oligonucleotide primed (DOP) polymerase chain reaction (PCR)-mediated DNA amplification were performed as described by Fiegler *et al*.[21] The PCR products were spotted onto Corning CMT-Gaps II glass slides using an Omnigrid microarrayer (Gene Machines, San Carlos, CA, USA). DNA labeling, hybridization and data acquisition was done as recently described.[10,18,19] The ratios of two hybridizations with reversed dye labeling were averaged and normalized. The diagnostic cut-off level for each individual experiment was determined by calculating the mean and subsequently using plus/minus three standard deviations of all clones from chromosomes 1 to 22.

Affymetrix GeneChip hybridization and statistical analysis of the microarray data

Affymetrix hybridization was carried out with the U133A GeneChips according to the manufacturer's recommendations, as previously described.[10] Probe intensities were normalized using a variance stabilization method.[22] Gene expression levels were estimated by fitting an additive model[23] employing a median polish routine. The significance of differential gene expression was analyzed with a two-step procedure, a global step and a gene-by-gene step. Global tests for differential gene expression were performed for predefined sets of genes using the ANCOVA test of Mansmann and Meister.[24] This test allows several factors to be included in a linear model and analyzed separately for differential gene expression. The resulting empirical permutation *p* values were adjusted for multiplicity using Bonferroni's correction since multiple chromosomes were tested at the same time. We included the ABC/GCB type of the lymphomas in the model when testing for the impact of a *MALT1* gain on gene expression. In order to determine lists of differentially expressed genes we performed a gene-by-gene analysis using the method of Smyth *et al*.[25] GCB and ABC sample labels were computed according to Wright *et al*.[26]

The data presented are available from the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (*www.ncbi.nlm.nih.gov/geo/*) through GEO accession number GSE4475 (the patients' identification numbers (MPI-number) are shown in *Online Supplementary Figure S1*).

Statistical analysis of genetic, histopathological, and clinical data

Mann-Whitney U, χ^2, Fisher's exact, and log-rank tests were applied to test for differences between groups. Survival was calculated from the day of diagnosis until death or the end of follow-up. Time-to-treatment-failure (TTTF) was calculated from the day of diagnosis until progression, relapse, start of salvage therapy, end of therapy without complete remission or death. A Cox proportional hazard model was employed to analyze prognostic factors. Since not all parameters of the International Prognostic Index were available for all cases, age at diagnosis and Ann Arbor stage were used to adjust for known prognostic factors.[27]

Table 1. Morphological, immunohistochemical, and genetic characteristics of 116 DLBCL with and without a gain of 18q21 including *MALT1*.

	Normal		*Gain of 18q21/MALT1*		*p**
CD10 ICH					
negative	44	(61%)	34	(77%)	0.056
positive	26	(36%)	8	(18%)	
n.a.	2	(3%)	2	(5%)	
BCL2 IHC					
negative	14	(19%)	0	(0%)	<0.001
pos.	57	(79%)	44	(100%)	
n.a.	1	(1%)	0	(0%)	
BCL6 IHC					
negative	12	(17%)	14	(32%)	0.104
positive	53	(74%)	27	(61%)	
n.a.	7	(10%)	3	(7%)	
Ki67-index					
<95	66	(92%)	34	(77%)	0.016
≥95	4	(6%)	10	(23%)	
n.a.	2	(3%)	0	(0%)	
MYC-partner					
IG-MYC	14	(19%)	3	(7%)	0.153
non-IG-MYC	5	(7%)	5	(11%)	
MYC negative	53	(74%)	35	(80%)	
n.a.	0	(0%)	1	(2%)	
IGH-BCL2-fusion					
negative	57	(79%)	41	(93%)	0.063
positive	15	(21%)	3	(7%)	
BCL6-break					
negative	49	(68%)	38	(86%)	0.069
positive	21	(29%)	6	(14%)	
n.a.	2	(3%)	0	(0%)	
Cell of origin					
ABC	12	(17%)	29	(66%)	<0.001
GCB	39	(54%)	10	(23%)	
unclassified	21	(29%)	5	(11%)	

**p values were calculated based on all cases that could be evaluated; n.a., not available; IHC, immunohistochemistry.*

Results

Interphase FISH and array CGH: the minimal overrepresented region on chromosome 18 lies in 18q21 and includes MALT1

According to array-CGH, the minimal overrepresented region on chromosome 18 was delineated to a 3.9 Mb region in 18q21.2 to 18q21.31 (clones RP11-7L24 to RP11-350K6) and encompassed 15 annotated genes including *MALT1*, but not *BCL2*. The telomeric

Table 2. Clinical characteristics of 81 DLBCL patients with and without a gain of 18q21/*MALT1*.

	All		*Normal*		*Gain of 18q21/MALT1*		*p**
Age							
<60	25	(31%)	18	(38%)	7	(21%)	
≥60	56	(69%)	30	(62%)	26	(79%)	0.146
Stage							
I or II	31	(38%)	21	(44%)	10	(30%)	
III or IV	41	(51%)	20	(42%)	21	(64%)	0.15
n.a.	9	(11%)	7	(15%)	2	(6%)	
Gender							
female	39	(48%)	23	(48%)	16	(48%)	
male	42	(52%)	25	(52%)	17	(52%)	1
Nodal/extranodal							
extranodal	11	(14%)	8	(17%)	3	(9%)	
nodal	38	(47%)	21	(44%)	17	(52%)	
nodal/extranodal	17	(21%)	8	(17%)	9	(27%)	0.482
n.a.	15	(19%)	11	(23%)	4	(12%)	
B-symptoms							
no	38	(47%)	24	(50%)	14	(42%)	
yes	28	(35%)	15	(31%)	13	(39%)	0.459
n.a.	15	(19%)	9	(19%)	6	(18%)	
Bone marrow involvement							
no	50	(62%)	30	(62%)	20	(61%)	
yes	10	(12%)	3	(6%)	7	(21%)	0.097
n.a.	21	(26%)	15	(31%)	6	(18%)	
Bulk							
no	25	(31%)	13	(27%)	12	(36%)	
yes	13	(16%)	5	(10%)	8	(24%)	0.506
n.a.	43	(53%)	30	(62%)	13	(39%)	
Chemotherapy							
B-ALL like	2	(2%)	1	(2%)	1	(3%)	
CHOP/COPBLAM-like	57	(70%)	35	(73%)	22	(67%)	
other	15	(19%)	8	(17%)	7	(21%)	0.79
n.a.	7	(9%)	4	(8%)	3	(9%)	
Radiation therapy							
no	52	(64%)	31	(65%)	21	(64%)	
yes	18	(22%)	10	(21%)	8	(24%)	0.787
n.a.	11	(14%)	7	(15%)	4	(12%)	
Rituxan							
no	55	(68%)	31	(65%)	24	(73%)	
yes	10	(12%)	6	(12%)	4	(12%)	1
n.a.	16	(20%)	11	(23%)	5	(15%)	
Lactate dehydrogenase							
low	17	(21%)	9	(19%)	8	(24%)	
high	23	(28%)	12	(25%)	11	(33%)	1
n.a.	41	(51%)	27	(56%)	14	(42%)	
Response to treatment							
complete remission	31	(38%)	22	(46%)	9	(27%)	
no change/ partial remission	10	(12%)	7	(15%)	3	(9%)	
progression	15	(19%)	4	(8%)	11	(33%)	0.14
n.a.	25	(31%)	15	(31%)	10	(30%)	

**p values were calculated based on all cases that could be evaluated ; n.a., not available.*

margin of this region was mainly defined by one case, which showed an amplification of 18q21 including *MALT1* and a normal *BCL2* status. The centromeric margin was defined by another case showing an amplification of 18q21 spanning exactly the minimal overrepresented region between clones RP11-7L24 and RP11-350K6 including *MALT1*. Interestingly, the region telomeric to RP11-350K6 including *BCL2* was gained but not amplified.

From the 40 cases with a 18q gain, 30 cases (75%) had a gain of 18q or part of it and ten (25%) had trisomy or polysomy of the whole chromosome.[18] According to FISH, the *MALT1* gene was gained in 44 of the 116 cases (38%). In all cases with a *MALT1* gain but one, a concomitant gain of *BCL2* was detected by interphase FISH and array-CGH. The latter case had *MALT1* amplification in 64% of the cells and a normal *BCL2* signal constellation (*see array-CGH results*). In the remaining cases, the signal numbers for *MALT1* and *BCL2* were similar, indicating a gain of the 18q21 region or part of it including both genes (further referred to as gain of 18q21/*MALT1*).

Among the 44 cases with a gain of *MALT1*, 28 (63%) showed mainly three copies, six (14%) showed four copies, six (14%) showed five copies, and four (9%) had an amplification of *MALT1*. The percentage of cells with a *MALT1* or *BCL2* gain ranged from 11% to 90%. Array-CGH results corresponded well with the interphase FISH results in 40 cases. In the remaining four cases, interphase FISH revealed a trisomy of *MALT1* and *BCL2* together with two signals for the *IGH*, *BCL6*, and MYC probes, but array-CGH showed a normal signal constellation for the *BCL2* and *MALT1* genes. In these four cases, the percentage of cells bearing three hybridization signals was 11%, 18%, 22%, and 40%. The relatively low proportion of cells carrying the abnormality was possibly the reason why these gains were not detected by array-CGH.[18] These cases were assigned as gain of 18q21. All cases without gains of *MALT1* and/or *BCL2* by FISH had normal array-CGH results for these regions.

The group without a 18q21/*MALT1* gain showed a higher frequency of *IGH-BCL2* fusions (p=0.06) and breaks in the *BCL6* locus (p=0.07), but the difference in frequency was not statistically significant (Table 1). Three cases with 18q21/ *MALT1* gains showed a *IGH-BCL2* fusion. In one case, array-CGH revealed a tetrasomy 18. In the other two cases, the gains involved the 18q12.1-18q21.33 and 18q21.1-18q21.33 regions, and the proximal border of the gain was telomeric of *BCL2* in both cases. Breaks targeting *MALT1* were not detected in any case.

Gain of 18q21 including MALT1 *correlates with an ABC-DLBCL expression signature and differential gene expression overlaps with that observed in ABC- vs. GCB comparisons*

Out of the 44 cases in the group with a 18q21/ *MALT1* gain, 29 (66%) showed an ABC signature and 10 (23%) had a GCB profile, whereas in the other 72 cases only 12 cases (17%) showed an ABC signature and 39 cases (54%) had a GCB pattern (p<0.001) (Table 1).

In order to determine differential gene expression between cases with and without a gain of 18q21/*MALT1*, we preceded in several steps. First, we applied a global test for differential expression between 18q21/*MALT1*-positive and 18q21/*MALT1*-negative patients, ignoring the ABC and GCB signatures. Global ANCOVA returned an empirical permutation p value of

0.004. Hence, there is differential gene expression between the two genetic subtypes of DLBCL. From a gene-by-gene test we obtained a list of 135 Affymetrix probe sets representing 98 differentially expressed genes with an estimated false discovery rate of 0.05. This list included *MALT1* and *BCL2* and some selected NF-κB target genes. In the next step, we included the GCB or ABC signature as a confounding factor in a full linear model using global ANCOVA. We restricted this part of the analysis to patients with an unambiguous GCB or ABC signature and excluded cases with an unclassified (type 3) signature. The global test for the influence of a 18q21/*MALT1* gain on gene expression gave a negative result (p=0.41). It is not, therefore, possible to distinguish 18q21/*MALT1*- specific from ABC-specific effects on gene expression.

Genomic gain of 18q21 including MALT1 is significantly associated with RNA upregulation of genes located on chromosome 18q including MALT1

Up to this point, we had only considered the global gene expression profiles looking at all probe sets at the same time. This represents a massive multiple testing problem (>22,000 array features) compromising the statistical power of the screening. In the following step we, therefore, increased the power of the test by including prior knowledge of the chromosomal location of the genes. We applied a global test of gene expression for each chromosome (excluding the X and Y chromosomes, 22 tests) separately and adjusted the 22 p values for multiple testing. This test identifies chromosomes with global changes in gene expression between the two groups. Interestingly, only chromosome 18 displayed global changes in gene expression, which were independent of the ABC/GCB status ($p_{adjusted}$<0.03). To further elucidate this finding, we analyzed the genes on chromosome 18 independently using a gene-by-gene test and identified 47 probe sets representing 33 differentially expressed genes with an estimated false discovery rate of 0.05. (*Online Supplementary Table S1 and Figure S1*). These differentially expressed genes included *MALT1, SERPINB8, NEDD4L, POLI, VPS4B, TXNL1, SMAD2, TCF4*, *SMAD4* and *PIK3C3*, but not *BCL2*.

Upregulation of NF-κB target genes in the group with a 18q21/MALT1 gain overlaps with ABC gene expression characteristics

Since *MALT1* is known to activate the NF-κB pathway and *BCL2* is transcriptionally regulated by NF-κB,[17] we assessed the differential expression of NF-κB target genes in the groups with and without a 18q21/*MALT1* gain. For this purpose, we selected all NF-κB target genes, which were previously reported by Feuerhake *et al.*[14] (*Online Supplementary Table S2)* and were present on the U133A-array used in this study (106 of the 116 probe sets reported, representing 68 genes). We subjected these 106 probe sets to a global test for differential gene expression[24] between cases with and without a 18q21 gain. We found that this set of genes together displayed global changes in gene expression (p<0.0001), if the ABC/GCB status, which overlaps with the presence of a 18q21 gain, was ignored. However, the significance was lost when the ABC/GCB status was taken into account as a confounding factor within the test (p=0.12). We cannot, therefore, distinguish between 18q21/*MALT1*- related and ABC-related effects on the expression of the analyzed NF-κB target genes.

Correlation with histopathology

In the group with a 18q21/*MALT1* gain, the histopathological panel review classified the DLBCL as follows: centroblastic variant (n=31; 71%), plasmablastic variant (n=4; 9%), immunoblastic variant (n=2; 4%), and anaplastic variant (n=1; 2%). In six cases (14%) a definitive assignment to one of the WHO DLBCL variants was not possible. In the cases without a 18q21/*MALT1* gain the following DLBCL variants were observed: centroblastic variant (n=46; 64%), immunoblastic variant (n=7; 10%), plasmablastic (n=3; 4%), anaplastic variant (n=2; 3%). In 14 DLBCL cases without a 18q21/*MALT1* gain (19%) a clear subgrouping according to the WHO classification was not possible.

All cases with a 18q21/*MALT1* gain showed BCL2 protein expression, whereas 79% of the other cases did so (p<0.001) (Table 1). In addition, the Ki-67 index, a marker of cell proliferation, was significantly higher in the group with a 18q21/*MALT1* gain (p=0.016) (Table 1). CD10 expression tended to be higher in the group without a 18q21/*MALT1* gain (p=0.056), which might be related to the higher frequency of GCB-type DLBCL. No significant difference was found in BCL6 protein expression (p=0.1) (Table 1).

Interestingly, among the cases with a 18q21/*MALT1* gain, four showed concomitant amplification of *MALT1* and *BCL2*. These cases were diagnosed as DLBCL centroblastic variant and did not differ morphologically or immunophenotypically from the other cases with a gain of 18q21/*MALT1*. One further case showing a *MALT1* amplification but normal *BCL2* status was detected and diagnosed as DLBCL centroblastic variant without evidence that this case represented a transformed marginal zone B-cell lymphoma.

Correlation with clinical parameters and prognosis: gain of 18q21 including MALT1 is an independent negative prognostic factor in DLBCL

Concerning known clinical risk factors, the patients in the group with a 18q21/*MALT1* gain showed a trend to more frequent bone marrow involvement (21% *vs.* 6%, p=0.097). There was no significant difference with respect to age, sex ratio, B-symptoms, bulky disease, extranodal involvement, lactate dehydrogenase level, or applied therapy including the use of rituxan (Table 2). In univariate analysis, we found a lower fraction of patients reaching a complete remission and a higher number of patients with progressive disease (p=0.014) in the group with a 18q21/*MALT1* gain (Table 2). Time-to-treatment-failure was not significantly shorter in the group with a 18q21/*MALT1* gain (p=0.12), but we found a trend to a shorter overall survival (5-year survival, 28% *vs.* 38%, p=0.09),

In the multivariate analysis incorporating age, Ann

Arbor stage, a break within the *MYC* gene, ABC/GCB gene expression subtype, and 18q21/*MALT1* status in all patients for whom information on these factors was available (n=71), the 18q21/*MALT1* status remained an independent negative prognostic factor for overall survival [hazard ratio 2.13 (95% CI: 1.08-4.23), p=0.03].

Discussion

The 116 DLBCL analyzed in our study were diagnosed according to the histopathological and immunohistochemical characteristics described in the WHO classification and additionally according to novel gene expression features recently defined by our group.[10]

Forty-three of our 116 cases of DLBCL had a genomic gain of *BCL2* and *MALT1* and one case revealed an amplification of *MALT1* together with a normal *BCL2* gene status (together 38%), while 72 cases (62%) had normal copy numbers of the *BCL2* and *MALT1* genes not considering changes in ploidy. Interestingly, gain of the *BCL2* gene was invariably associated with gain of the *MALT1* gene in our series. Moreover, the presence of a 18q21/*MALT1* gain correlated with upregulation of genes located on 18q including *MALT1* at the RNA level, independently of the ABC or GCB gene expression signature, and was strongly associated with overexpression of BCL2 protein. We found a significant accumulation of cases with a 18q21/*MALT1* gain among those with an ABC signature and 98 differentially expressed genes between the two genetic groups of DLBCL, including some selected NF-κB target genes. However, when we included the ABC/GCB signature as a confounding factor into the test, the influence of the 18q21/*MALT1* gain on gene expression was no longer significant. The overlap of ABC and 18q21 gains complicated efforts to distinguish effects on gene expression specific to ABC status or 18q21 gains. Linear models generated in all steps of analysis allowed us to test for differential gene expression (18q21 gain *vs.* normal) while taking into account the presence of an additional confounding factor (ABC *vs.* GCB signature). We interpret this by the unequal distribution of the DLBCL cases with and without 18q21/*MALT1* gains in the ABC- and GCB-categories. As a result, we could not distinguish 18q21/*MALT1*- specific from ABC-specific effects on gene expression.

BCL2 and *MALT1* are both located on chromosome 18q21, about 5 Mb apart from each other, and are both key genes in the pathogenesis of different subtypes of non-Hodgkin's lymphomas. *MALT1* codes for a paracaspase, a scaffold protein recently proposed to be related to selective control of the c-Rel subunit in the canonical NF-κB pathway.[28] The API2-MALT1 fusion protein resulting from the t(11;18)(q21;q21)[29-31] and the overexpressed MALT1 protein in another *MALT1*-associated translocation in MALT lymphomas, the t(14;18)(q32;q21),[32,33] have been shown to activate NF-κB and thereby promote cellular proliferation and resistance to apoptosis.[34-36] In addition to the two translocations, amplifications and gains of *MALT1* have been described in different subtypes of non-Hodgkin's lymphomas,[32,37] but their pathophysiological significance is not known.

BCL2 is a gene involved in the regulation of apoptosis and plays a major role in the response of malignant cells to a variety of stresses that may lead to apoptosis, including chemotherapy.[38] One major mechanism leading to BCL2 protein overexpression is the t(14;18)(q32;q21) resulting in juxtaposition of the *BCL2* gene and the *IGH* locus. The t(14;18) is found in most follicular lymphomas and about 20% of DLBCL.[11,39] The t(14;18) occurs mainly in GCB-DLBCL, being present in approximately 35% of these cases. In GCB-DLBCL, the t(14;18) correlates with BCL2 mRNA and protein expression,[39] indicating that this is the major mechanism in upregulation of BCL2 expression in this subgroup.

However, ABC-DLBCL also have high levels of BCL2 expression in the absence of t(14;18), indicating alternative mechanisms of BCL2 upregulation. Using interphase FISH, Iqbal *et al*. recently showed that BCL2 protein expression in ABC-DLBCL is significantly associated with genomic gain of *BCL2*.[16] In another study, gain of 18q21-22 was detected by CGH in one third of ABC-DLBCL, but less frequently in GCB-DLBCL (10%) and PM-DLBCL (16%).[40] Our study revealed compatible results with an accumulation of cases with a gain or amplification of the *BCL2* and *MALT1* genes in the ABC subtype as well as a highly significant association of these gains with BCL2 RNA and protein expression.

We additionally showed that a gain of *BCL2* was accompanied by a gain of *MALT1* in all of our cases of DLBCL. In line with this observation, gain of *BCL2* was associated with gain of *MALT1* in one study analyzing lymphoma cell lines by array-CGH and in a further study using array-CGH and quantitative PCR to evaluate DLBCL patients with chromosomal gains in 18q21-q22.[32,40] Most other studies applied conventional CGH analysis or interphase FISH with *BCL2*-specific probes. It is not, therefore, possible to assess in these studies whether or not *MALT1* was gained in addition to *BCL2*.[16,41] Despite the scarcity of data, the frequent association of genomic gains of *BCL2* and *MALT1* might suggest a cooperative action of these two and possibly additional 18q21 genes in DLBCL.

Given that *BCL2* is transcriptionally regulated by NF-κB[17] and NF-κB is activated by *MALT1*, which was found to be upregulated on genomic and RNA levels in our study, we evaluated the differential expression of 68 selected NF-κB target genes in our DLBCL cases. There were significant changes in the expression of this set of genes between the two *MALT1* genetic groups; however, the significance was lost when ABC/GCB status was included as a confounding factor in the test. Hence, also for the analyzed NF-κB target genes, it was not possible to distinguish between 18q21/*MALT1*- and ABC-related effects on their expression.

In contrast to the analyzed NF-κB target genes and the global differential gene expression between the two genetic groups, the differential expression of *MALT1* itself and 32 other genes located on chromosome 18q was independent of the gene expression subtype. We focused our investigations on the *MALT1* gene, but can-

not exclude that any of the other 32 genes may play a pathogenetic role in DLBCL cases with a 18q21 gain. The pathogenetic mechanism of action of *MALT1* and the other 18q genes in this context cannot be concluded from our data.

We found that gain of 18q21 including *MALT1* and *BCL2* was an independent negative prognostic factor in our cases of DLBCL, although the relatively small number of patients and the heterogeneous treatment protocols applied should be taken into account when interpreting this result. In the multivariate analysis including age, Ann Arbor stage, a break within the *MYC* gene, ABC/GCB gene expression subtype, and 18q21/*MALT1* status, 18q21/*MALT1* status was a statistically significant independent negative prognostic factor for overall survival (hazard ratio 2.13, p=0.03). In the univariate analysis, response to treatment was significantly reduced (p=0.014) and a trend to a shorter overall survival (p=0.09) was demonstrated.

Bea *et al.* found overrepresentation of 18q in DLBCL to be significantly associated with a primary nodal presentation, high serum lactate dehydrogenase, and high International Prognostic Index score.[41] In addition, 18q gains were more commonly found in tumors with a higher number of chromosomal imbalances.[41] Similar to the present study, patients with 18q gain had a significantly shorter cause-specific overall survival, higher risk of relapse, and a tendency to lack a complete response to treatment.[41] This relationship between 18q gains and a more aggressive clinical behavior in DLBCL had not been well recognized previously. A recent study on follicular lymphoma identified a distinct clinical presentation in patients with 18q gains, although no differences were observed in outcome.[42] In another study, dup(18q) was found to predict an unfavorable overall survival in follicular lymphomas with the t(14;18).[43] In surgically resected t(11;18)-negative gastrointestinal DLBCL, trisomy of 18q21, as shown by interphase FISH, was also associated with a shorter overall survival.[44]

The various reports in the literature on the t(14;18) or BCL2 expression as a prognostic indicator in DLBCL have been inconclusive.[45-49] Interestingly, a recent study showed that BCL2 protein expression represents a negative prognostic factor in the ABC-DLBCL subgroup, but not in the GCB subgroup.[16] In the latter study, BCL2 protein expression in the ABC-DLBCL subgroup was associated with amplification and gain of the *BCL2* gene,[16] which was also demonstrated in the present study. Since 18q21/*MALT1*-positive cases are frequently found in the ABC-DLBCL subgroup, it cannot be excluded that this prognostically unfavorable gene expression subtype had an influence on the clinical course in our study.

In summary, our data demonstrate that a gain of 18q21 including *MALT1* is associated with differential RNA expression of genes located on 18q, independently of the gene expression subtype, as well as BCL2 protein expression and clustering in the ABC subtype. The differences in expression of genes not located on 18q, including the analyzed NF-κB target genes, overlapped with the ABC/GCB, gene expression subtype. So, the individual influence of the gene expression signature and the 18q21/*MALT1* status on gene expression cannot be viewed separately. Finally, 18q21/*MALT1* gain may be a prognostic indicator in DLBCL, although this needs further evaluation in a prospective trial with homogeneously treated patients combining genomic analysis and gene expression profiling.

Authorship and Disclosures

JD and EMMP contributed equally to the study and should both be regarded as first authors; JD, EMMP, and RSi: conceived, designed and performed research, collected and analyzed data, wrote the manuscript; SB: analyzed and interpreted data, wrote the manuscript; SW and CS: performed research, collected and analyzed data, wrote the manuscript; HB: collected and analyzed data, wrote the manuscript; MH: provided material, collected and analyzed data, wrote the manuscript; WK and DL: performed research and collected data; AR: conceived and designed research, provided material, performed research, collected and analyzed data; EH: provided material, performed research, collected and analyzed data; GO and HS: provided material, collected data; SBG: provided material; PM: provided material, wrote the manuscript; ML and RSp: analyzed data, wrote the manuscript; LT: collected and analyzed data All authors approved the final version of the manuscript. The authors reported no potential conflicts of interest.

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