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Impact of Single or Combined Genomic Alterations of *TP53*, *MYC*, and *BCL2* on Survival of Patients With Diffuse Large B-Cell Lymphomas

A Retrospective Cohort Study

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Abstract: *MYC* and *BCL2* translocations as well as *TP53* deletion/mutation are known risk factors in diffuse large B-cell lymphoma (DLBCL) but their interplay is not well understood.

In this retrospective cohort study, we evaluated the combined prognostic impact of *TP53* deletion and mutation status, *MYC* and *BCL2* genomic breaks in tumor samples of 101 DLBCL patients. The cohort included 53 cases with *MYC* rearrangements (*MYC*+).

TP53 deletions/mutations (*TP53*+) were found in 32 of 101 lymphomas and were equally distributed between *MYC*+ and *MYC*− cases (35.8% vs. 27.1%). *TP53*+ lymphomas had lower responses to treatment than *TP53*− (complete remission 34.4% vs. 60.9%; $P = 0.01$). *TP53* alteration was the dominant independent prognostic factor in multivariate analysis ($P = 0.01$). Overall survival (OS) varied considerably between subgroups with different genomic alterations: Patients with sole *MYC* translocation, and interestingly, with triple *MYC*+/*BCL2*+/*TP53*+ aberration had favorable outcomes (median OS not reached) similar to patients without genomic alterations (median OS 65 months). In contrast, patients with *MYC*+/*BCL2*+/*TP53*− double-hit lymphomas (DHL) (28 months), *MYC*+/*BCL2*−/*TP53*+ lymphomas (10 months) or sole TP53 mutation/deletion (12 months) had a poor median OS. Our findings demonstrate differences in OS of DLBCL patients depending on absence or presence of single or combined genetic alterations of *MYC*, *BCL2*, and *TP53*. Cooccurrence of *TP53* and *BCL2* aberrations ameliorated the poor prognostic impact of single *TP53*+ or *BCL2*+ in *MYC* positive patients.

This pilot study generates evidence for the complex interplay between the alterations of genetic pathways in DLBCL, which goes beyond the concept of DHL. The variable survival of DLBCL patients dependent on single or combined alterations in the *TP53*, *MYC*, and *BCL2* genes indicates the need for comprehensive genomic diagnosis.

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Abbreviations: 95% CI = 95% confidence interval, *BCL2* = B-cell CLL/lymphoma 2, *BCL6* = B-cell CLL/lymphoma 6, BCLU = B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma, BL = Burkitt lymphoma, BM = bone marrow, COO = cell of origin, CR = complete remission, CS = clinical stage, DH = double-hit, DHL = double-hit lymphoma, DHS = double-hit score, DLBCL = diffuse large B-cell lymphoma, DNA = desoxyribonucleic acid, EPOCH-R = etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone and rituximab, FISH = fluorescence in situ hybridization, GCB = germinal center like, HD-MTX = high-dose methotrexate, HIV = human immunodeficiency virus, HR = hazard ratio, *IGH* = immunoglobulin heavy locus, IHC = immunohistochemistry, IPI = International Prognostic Index, LDH = lactate dehydrogenase, *MYC* = v-myc avian myelocytomatosis viral oncogene homolog gene, nGCB = non-germinal center like, OS = overall survival, *P* = *P*-value, PD = progressive disease, PR = partial remission, R-CHOP = rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone, SD = stable disease, TMA = tissue microarray, *TP53* = tumor protein p53.

INTRODUCTION

Clinical risk stratification and treatment decisions in diffuse large B-cell lymphoma (DLBCL) are still based on the International Prognostic Index (IPI).[1] However, increasing evidence suggests that the prognosis is strongly dependent on

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concomitant genetic alterations.[2–6] Many studies verified the importance of translocations of the *MYC* and *BCL2* genes as well as mutations or deletions of the *TP53* gene.[7–14] However, the clinical impact of simultaneous occurrence of these genetic changes is not well understood, even in the era of whole genome sequencing.[15–20]

TP53 plays an important role in regulation of cell cycle and cell proliferation. Based on its capability to induce apoptosis upon desoxyribonucleic acid (DNA) damage it acts as a tumor suppressor. Mutations in the *TP53* gene abrogate genetic stability and lead to uncontrolled proliferation of oncogene driven tumor cells. *TP53* mutations in aggressive B-cell lymphomas are found at frequencies of 33% in Burkitt lymphoma (BL), 21% to 23% in DLBCL, and 29% to 80% in transformed follicular lymphoma.[8,9,21] The association of *TP53* mutation with inferior overall survival (OS), transformation into aggressive lymphoma and resistance to chemotherapy has been reported[21–24]; however, recent research often focuses on genetic changes of *MYC*, *BCL2*, and *BCL6*.[3,10,13,25,26]

The *MYC* translocation is the hallmark of BL. The balanced translocation between the *MYC* locus (8q24) and an immunoglobulin gene, most commonly *IGH* (14q32) results in overexpression of the MYC protein. Albeit being the hallmark of BL, *MYC* translocations are also found with a frequency of 5% to 15% in DLBCL and in 50% of B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma (BCLU).[27] These aggressive B-cell lymphomas have a poor outcome in many studies.[3,4,28,29] Lymphomas with concurrent *MYC* and *BCL2* translocations (double-hit lymphoma, DHL) as well as triple hit lymphomas with additional breaks including the *BCL6* gene have been investigated in many studies.[3,7,10,11,17,25,30–32] DHL are associated with aggressive, often widespread extranodal disease, dismal prognosis, are often refractory to standard chemotherapy[13,28,29,33,34] and represent a distinct entity. An overexpression of MYC, BCL2 protein regardless of the underlying genetic hit has also been identified as poor prognostic factor[30]; however, reported results are not uniform.[30,35,36]

Despite of the increasing focus on DHL so far, only few studies included the *TP53* status in this setting.[11,12,37] We have previously studied the interaction of *MYC*, *BCL2*, and *TP53* in a mouse model and in a small number of patients with BL and DLBCL.[38]

Here we investigated the prognostic value of *TP53* deletions and mutations in patients with and without *MYC* and/or *BCL2* structural aberrations in a large retrospective series of patients treated with immunochemotherapy. The study provides novel insights into the complex interplay of *TP53*, *MYC*, and *BCL2* alterations in aggressive lymphomas.

METHODS

For this retrospective study we analyzed 2 similar-sized DLBCL cohorts with and without *MYC* break (N = 53 vs. 48). Inclusion criteria were: 18 years of age or older, known medical history, diagnosed and treated at one of the participating institutions, rituximab containing treatment was administered. Transplant and human immunodeficiency virus (HIV) associated lymphomas were excluded. Clinical and demographical data were collected. Clinical data included previous medical history, date of diagnosis, histological subtypes, art and duration (including number of cycles) treatments, date and quality of response, date of relapse and death, blood tests and observation time.

In a first step patients diagnosed at the Medical University of Vienna with known *MYC* status at diagnosis were selected (N = 34). Additional 9 cases with *MYC* translocation were contributed from the Portuguese Institute of Oncology, Lisbon and 7 from other Austrian hospitals. HIV associated lymphomas were not included.

In a second step, 51 cases of the Medical University of Vienna with available tissue were screened for *MYC* translocation retrospectively, 3 were positive. The 48 *MYC* negative patients comprised the second group.

In a third step, *BCL2* and *TP53* fluorescence in situ hybridization (FISH) as well as *TP53* sequencing has been performed.

For final analysis, only patients with information about all three (*MYC*, *BCL2*, and *TP53*) genes were included. Lymphomas were designated as "positive" on the basis of genetic results (*MYC*+: translocations; *BCL2*+: translocations; *TP53*+: deletions and/or mutations) regardless of protein expression.

All cases were reviewed by 2 independent pathologists and diagnoses were adjusted according to the WHO Classification 2008,[39] cell of origin (COO) has been defined according to the Hans algorithm.

The final cohort consisted of 101 DLBCL (including 16 BCLU) treated with rituximab containing regimens. Patient's clinical and demographical characteristics are presented in Table 1. Patients were followed until January 2015 or until death, whatever occurred first. Analysis was performed after availability of molecular information. Mean observation period (date of diagnosis to last follow up) was 27.45 months (range 1–128 months; standard deviation 28.22 months).

Ethical approval was granted by the Ethics Committee of the Medical University of Vienna (#1051/2013).

Immunohistochemistry (IHC)

IHC was done on whole tissue sections (N = 64) and on sections from tissue microarrays (TMA; N = 37) consisting of 3 representative 0.8 mm cores of tumor tissue. IHC was performed on formalin fixed, paraffin-embedded sections on the automated Leica Bond III Immunostainer (Leica Biosystems, Nussloch, Germany) using routine protocols. According to Kaserer et al,[40] TP53 staining (P53 antibody Clone DO-7, DAKO, Glostrup, Denmark) was interpreted as positive if at least 30% of nuclei of tumor cells showed a strong or moderate staining and when there was a sheet like, diffuse staining pattern within the whole tumor tissue or at least within parts of the tumor tissue.

For BCL2 (N = 98) and MYC (N = 83) staining, the following antibodies were used: BCL2 (Clone 124, DAKO), MYC (Clone Y69, Epitomics, Burlingame, CA). Cut-off values for BCL2 and MYC were 50% and 40%, respectively.[10] Samples with weakly stained tumor cells were interpreted as negative, regardless of the percentage of positively stained cells.

Interphase FISH Analysis

FISH was done on formalin-fixed, paraffin-embedded tissue except in 18 cases, where fresh tissue was available. Following probes were used: LSI MYC Dual Color, Break Apart Rearrangement Probe (Vysis, Downer's Grove, LSI IGH/BCL-2 Dual Color, Dual Fusion Translocation Probe (Vysis) LSI TP53 (17q13.1) Single Color probe and centromere-17-specific probe (Vysis).[41] In each case a total of 200 interphase nuclei was counted. The cut-off for positivity was determined at 10% of nuclei showing aberrant hybridization signals).

TABLE 1. Clinical and Genetic Patient Characteristics

	Whole Cohort		TP53 Positive		TP53 Negative			MYC Positive		MYC Negative		
Characteristics	**No.**	**%**	**No.**	**%**	**No.**	**%**	***P***	**No.**	**%**	**No.**	**%**	***P***
	101		32	31.7	69	68.3		53	52.5	48	47.5	
Gender							0.99					0.42
Female	41	40.6	13	40.6	28	40.6		24	45.3	17	35.4	
Male	60	59.4	19	59.4	41	59.4		29	54.7	31	64.6	
Age > 60 years	48	47.5	15	46.9	33	47.8	0.93	**19**	**35.8**	**29**	**60.4**	**0.02**
Stage = III–IV	67	66.3	20	62.5	47	68.1	0.51	35	66.0	32	66.7	0.84
IPI = 3–5	60	59.4	20	62.5	40	58.0	0.85	32	60.4	28	58.3	0.86
Genetic aberration	70	69.3	32	100	38	55.1		53	100	17	35.4	
MYC+	53	52.5	19	59.4	34	49.3	0.34	53	100	0	0	NA
GCB	44	83.0										
nGCB	7	13.2										
BCL2+	28	27.7	9	28.1	19	27.5	0.95	**22**	**41.5**	**6**	**12.5**	**0.002**
GCB	26	92.9										
nGCB	2	7.1										
TP53+	32	31.7	32	100	0	0	NA	19	35.8	13	27.1	0.39
GCB	21	65.6										
nGCB	10	31.1										
Triple−	31	30.7	0	0	31	44.9	NA	0	0	31	64.6	NA
GCB	11	35.3										
nGCB	20	64.5										
Sole *BCL2*+	4	4.0	0	0	4	5.8	NA	0	0	4	8.3	NA
GCB	3	75.0										
nGCB	1	25.0										
Sole *TP53*+	11	10.9	11	34.4	0	0	NA	0	0	11	22.9	NA
GCB	4	36.4										
nGCB	7	63.3										
BCL2+*TP53*+	2	2.0	2	6.3	0	0	NA	0	0	2	4.2	NA
GCB	2	100										
nGCB	0											
Sole *MYC*+	19	18.8	0	0	19	27.5	NA	19	35.8	0	0	NA
GCB	14	73.3										
nGCB	4	21.1										
MYC+*BCL2*+	15	14.9	0	0	15	21.7	NA	15	28.3	0	0	NA
GCB	15	100										
nGCB	0											
MYC+*TP53*+	12	11.9	12	37.5	0	0	NA	12	22.6	0	0	NA
GCB	9	75.0										
nGCB	2	16.7										
Triple+	7	6.9	7	21.9	0	0	NA	7	31.2	0	0	NA
GCB	6	85.7										
nGCB	1	14.3										
BM infiltration	24	23.8	8	25.0	16	23.2	0.89	**17**	**32.1**	**7**	**14.6**	**0.03**
High LDH	70	69.3	23	71.9	47	68.1	0.58	38	71.7	32	66.7	0.36
Extranodal	76	78.4	27	84.4	49	71.0	0.15	40	75.5	36	75.0	0.81
Transformation	8	7.9	1	3.1	7	10.1	0.26	5	9.4	3	6.3	0.46
COO							0.66					**<0.001**
nGCB	35	34.7	10	31.3	25	36.2		**7**	**13.2**	**28**	**58.3**	
GCB	64	64.4	21	65.6	43	62.3		**44**	**83.0**	**20**	**41.7**	
NA	2	2.0	1	3.1	1	1.4		2	3.8	0	0	
Response							**0.01**					0.74
CR	53	**52.5**	**11**	**34.4**	**42**	**60.9**		26	49.1	26	56.3	
PR	17	26.8	4	12.5	13	18.8		7	13.2	8	20.8	
SD	2	2.0	2	6.3	0	0		1	1.9	1	2.1	
PD	22	21.8	11	34.4	11	15.9		13	24.5	9	18.8	
NA	7	6.9	4	12.5	3	4.3		6	11.3	1	2.1	
Death	42	**42.4**	**18**	**56.3**	**24**	**34.8**	**0.03**	21	41.2	21	43.8	0.80

BCL2+ = presence of *BCL2* translocation; BM = bone marrow; COO = cell of origin; CR = complete remission; GCB = germinal center B-cell like; high LDH = LDH levels above the reference cut-off of 245 U/l; IPI = International Prognostic Index; *MYC*+ = presence of *MYC* translocation; NA = not applicable; nGCB = nongerminal center B-cell like; PD = progressive disease; *P* = *P*-value; PR = partial remission; SD = stable disease; *TP53*+ = presence of *TP53* deletion/mutation; triple− = absence of *MYC* translocation and *BCL2* translocation and *TP53* deletion/mutation; triple+ = presence of *MYC* translocation and *BCL2* translocation and *TP53* deletion/mutation.

Significant differences are indicated in bold letters.

Sequencing of *TP53*

TP53 mutation is associated with increased TP53 protein expression[42] and *TP53* mutational status was assessed in all TP53 IHC positive cases. *TP53* sequencing was performed using ABI Big dye terminator version 1.1 cycle sequencing kit as described previously.[38]

Sequencing analyses covered exons 4 to 11 and flanking intron regions. Purified DNA fragments were run on an ABI 330 Genetic Analyzer. Sequences were analyzed using the SeqScape analysis software program Versions 2.5 and 2.7 (Applied Biosystems/Life Technologies; Carlsbad, California, USA).

Statistical Methods

Statistical analysis was performed using IBM SPSS Statistics 21.0 (IBM Corporation, Armonk, NY) software. Statistical methods comprised the Chi-square analysis or Fisher exact test to compare baseline characteristics, Kaplan–Meier estimates for survival functions, log-rank test for a comparison of survival distributions and multivariate Cox proportional hazards regression models for the identification of significant and independent prognostic factors for OS. *P* values (*P*) of <0.05 (2-sided) were considered statistically significant.

The endpoints were to investigate the incidence of sole/combined genetic aberrations, to characterize clinical and demographic features of the sub-sets as well as to investigate the prognostic influence of sole/combined genetic aberrations in the whole cohort and in the sub-sets.

First, patient's clinical and demographical characteristics were collected. Then all patients with or without *MYC*-translocation and in a second step the 2 sub-sets with or without *TP53* aberrations were compared using the Chi-square analysis or Fisher exact test.

Second, the influence of clinical and molecular parameters on OS was determined using Kaplan–Meier OS curves and the log-rank test. Univariate and multivariate analysis were performed using Cox regression models.

Covariates available for analysis were presence or absence of *MYC*+, *BCL*+, and *TP53*+ (regardless of combined aberrations), age, serum lactate dehydrogenase levels (LDH) (U/ml), bone marrow (BM) infiltration (numeric) and factor variables such as sex, age-group (>60 years or younger), IPI high (3–5) versus low (1–2), LDH high versus low, presence or absence of BM infiltration, localization (nodal/extranodal), transformation, COO, and first-line therapy. BM infiltration and LDH were considered as numeric as well as factor variables. Transformation and combined genetic aberrations were excluded from further considerations in the Cox models due to a not sufficient number of cases.

RESULTS

TP53 Deletions/Mutations

TP53 FISH and TP53 IHC were performed in all samples. FISH analysis revealed a *TP53* heterozygote deletion in 12 cases (11.9%). Patient samples with positive IHC were selected for further sequencing (N = 49). Missense mutations were found in 29 tumor samples (28.7%). Altogether, 32 (31.7%) patients had *TP53* deletions and/or mutations. The majority of these patients had *TP53* mutation only (N = 20, 19.8%), 9 (8.9%) patients had both a deletion and a mutation on the remaining allele, and 3 patients (3%) had a deletion but no material available for sequencing (Supplementary Table 1, http://links.lww.com/MD/A584). *TP53* mutations are displayed in Supplementary Table 1, http://links.lww.com/MD/A584.

There were no major differences in the characteristics of patients with or without *TP53* alteration at diagnosis regarding sex, age, clinical stage (CS), IPI, BM infiltration, LDH, COO (Table 1). Standard rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) therapy and high-dose methotrexate (HD-MTX) containing treatments were equally used in *TP53*+ and *TP53*− patients.

Patients with *TP53*+ showed significantly more primary resistance to treatment (complete remission (CR) rate 34.4% vs. 60.9%, progressive disease (PD) 34.4% vs. 15.9%) ($P = 0.01$). Patients with a *TP53*+ lymphoma showed a significantly shorter OS (median 12 months vs. not reached; hazard ratio (HR) 2.206, 95% confidence interval (95% CI) 1.183–4.117; $P = 0.01$) (Table 2; Figure 1A).

Relationship Between *MYC* Translocations and *TP53* Deletions/Mutations

MYC+ cases were similarly distributed between the *TP53*+ and *TP53*− cohorts (59.4% vs. 49.3%). Vice versa, there were 35.8% *TP53*+ cases in the *MYC*+ cohort versus 27.1% in *MYC*− patients (Table 1). These data indicate that *TP53*+ occur at similar frequency in lymphomas irrespective of the absence or presence of *MYC*+.

We next compared the impact of *TP53* status on survival of *MYC*+ *or* − patients. *TP53*+ patients without *MYC*+ had a significantly shorter OS (median OS 12 months vs. 65 months; HR 3.510, 95% CI 1.374–8.967; $P = 0.01$) (Figure 1B). Interestingly, there was no significant impact of *TP53*+ in the *MYC*+ group on OS (median 10 months vs. not reached; HR 1.840, 95% CI 0.759–4.461; $P = 0.18$) (Figure 1C). We noted that a considerable number of combined *TP53*+/*MYC*+ lymphomas were also *BCL2*+ (triple+) (N = 7, 13.2%) (Table 1). This observation together with the loss of impact of *TP53*+ in

TABLE 2. Uni- and Multivariate Analyses Determining the Influence of Clinical and Molecular Parameters at Diagnosis on Overall Survival of 101 Patients

	Univariate Analyses			Multivariate Analyses		
	P	**HR**	**95% CI**	***P***	**HR**	**95% CI**
IPI = 3–5	**0.01**	**2.570**	**1.235–5.439**	**0.02**	**2.358**	**1.137–4.891**
High LDH	0.06	2.117	0.976–4.592			
BM	**0.03**	**2.200**	**1.092–4.433**	0.32		
Extranodal	0.34	1.486	0.655–3.371			
Gender	0.81	0.925	0.497–1.722			
Age > 60 years	0.09	1.696	0.919–3.132			
TP53+	**0.01**	**2.206**	**1.183–4.117**	**0.01**	**2.358**	**1.240–4.555**
MYC+	0.90	0.963	0.518–1.799			
BLC2+	0.65	1.166	0.604–2.247			
1st line therapy	0.99	0.999	0.525–1.899			

95% CI, 95% confidence interval; *BCL2*+, presence of *BCL2* translocation; BM, presence of BM infiltration; Extranodal, extranodal manifestation; high LDH, LDH levels above the reference cut-off of 245 U/l; HR, hazard ratio; IPI, International Prognostic Index; *MYC*+, presence of *MYC* translocation; *P*, *P*-value; *TP53*+, presence of *TP53* deletion/mutation.

COX regression models were used; *P*-values, HR, and 95% CI are listed. Significant differences are indicated in bold letters.

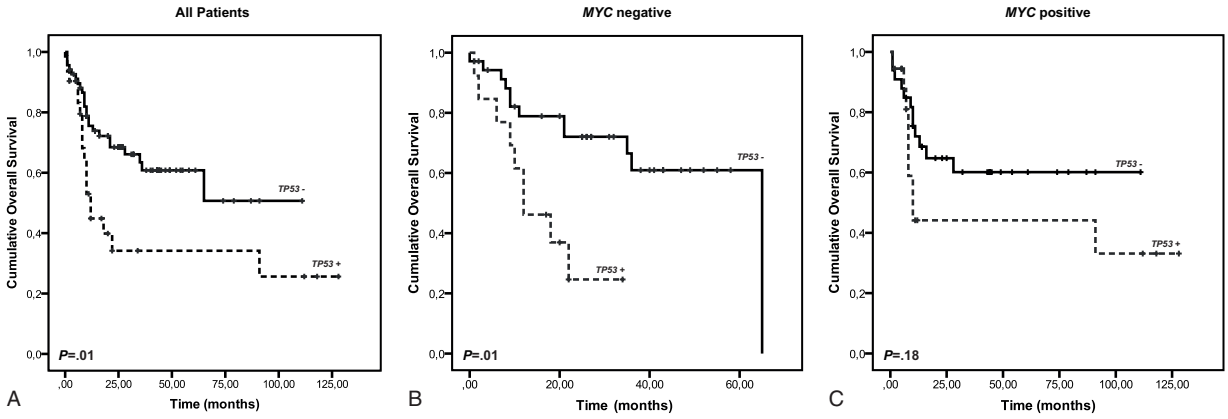

FIGURE 1. *TP53* alteration is associated with significant shorter overall survival in the whole cohort while its prognostic value is attenuated in patients with *MYC* translocation. Kaplan–Meier plots showing (A) overall survival of all patients (N = 101) according to *TP53* deletion/mutation; (B) overall survival of *MYC* negative patients (N = 48) according to *TP53* deletion/mutation; and (C) overall survival of *MYC* positive patients (N = 53) according to *TP53* deletion/mutation. The negative prognostic impact of *TP53* alteration is reduced and loses significance in patients with a concurrent *MYC* translocation. *P*: *P*-value.

MYC+ patients prompted us to perform a further subgroup analysis including *BCL2*+.

Relationship Between *BCL2* Translocations and *TP53* Mutation/Deletions

BCL2+ were detected in 28 patients (27.7%). They rarely occurred alone (4/28) but mainly in conjunction with additional aberrations. *BCL2*+ were frequently associated with *MYC*+ lymphomas (DHL: 15/28), followed by *TP53*+ and *MYC*+ (7/28) and *TP53*+ (2/28). They were evenly distributed between *TP53*+ (9/32, 28.1%) and *TP53*− (19/69, 27.5%) patients (Table 1). *BCL2/TP53* directed analysis revealed a differential clinical outcome in the *TP53*+ and *TP53*− subgroups. In *TP53*− patients, *BCL2*+ had a poor prognostic impact (median OS 28 months vs. not reached; HR 2.439, 95% CI 1.065–5.582; $P = 0.04$) (Figure 2A). Interestingly, in *TP53*+ patients an additional *BCL2*+ even reversed prognosis indicating an equalizing effect of the two genetic defects (median OS not reached vs. 10 months; HR 0.308, 95% CI 0.086–1.102; $P = 0.07$) (Figure 2B).

Outcome of Patients According to Combined *TP53*, *MYC*, and *BCL2* Genetic Analysis

A similarly unexpected result was observed when *MYC*+ patients were analyzed for OS in relation to *TP53* and *BCL2*. Patients with sole *MYC*+ *TP53*−/*BCL2*− (N = 18) had the best OS (median not reached) (Figure 2C). However, the OS of the 7 patients with a triple *MYC*+/*TP53*+/*BCL2*+ aberration was almost equally favorable (median OS not reached). In contrast, patients with *MYC*+/*BCL2*+ or *MYC*+/*TP53*+ had a shorter median survival of 28 or 10 months, respectively. Patients with sole *TP53*+ (N = 11) had an OS of 12 months while patients without any of the 3 alterations (N = 31) had an OS of 65 months (data not shown).

Univariate and Multivariate Analysis of Clinical and Genetic Factors

Results of univariate analysis for OS are shown in Table 2. In multivariate analysis high IPI (HR 2.358, 95% CI 1.137–4.891; $P = 0.02$) and *TP53*+ (HR 2.358, 95% CI 1.240–4.555; $P = 0.01$) remained independent poor prognostic factors.

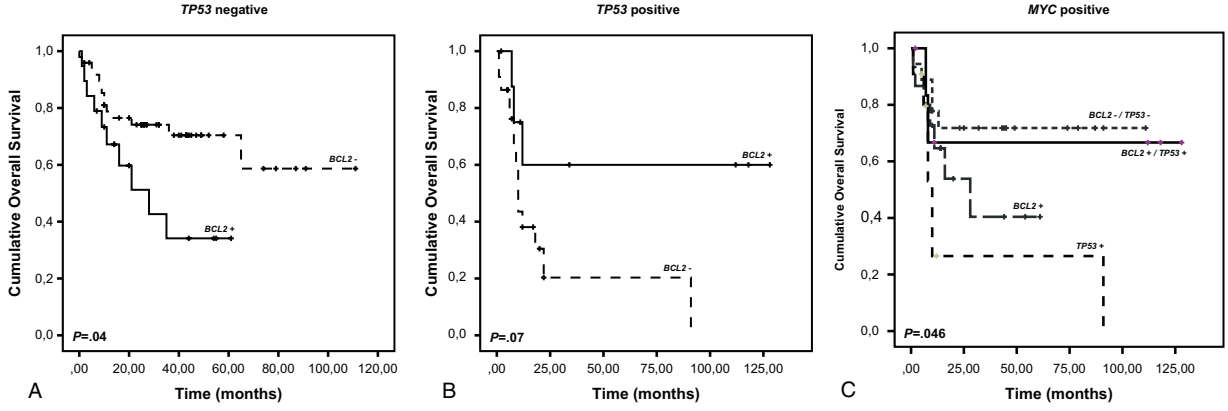

FIGURE 2. Additional *BCL2* translocation abrogates the negative prognostic impact of *TP53* aberration. Overall survival of (A) *TP53* negative patients (N = 69) according to *BCL2* translocations; (B) *TP53* positive patients (N = 32) according to *BCL2* translocations; The presence of *BCL2* translocation has opposite impact on overall survival in *TP53* negative (inferior) and positive (superior) patients; (C) *MYC* positive patients (N = 53) according to combined genetic aberrations. Patients with cooccurrence of *TP53, BCL2* and *MYC* aberrations (triple-hit) have a good prognosis. *P*: *P*-value.

Because of the small sample size and low statistic power a Cox regression model describing the interaction between *BCL2*+ and *TP53*+ was performed in which the reversal of relative risk was also seen in the combination (Table 3). This effect remained after adjustment for IPI.

Double-Hit Protein Score (DHS) in Molecular Double-Hit and Triple-Hit Lymphomas

Protein staining for all MYC, BCL2, and TP53 was available in 80 samples. Nine (60%) of the DHL had also a protein expression for both MYC and BCL2 (DHS 2), 3 patients (20%) had a DHS of 1 (2 patients due to BCL2 expression, 1 due to MYC expression), the other 3 patients were positively stained for BCL2 protein, but MYC protein staining was not available. Among the triple-hit lymphomas 4 patients (57.14%) were with DHS of 2, 2 (14.29%) with DHS2 and 1 patient had BCL2 protein, but MYC staining was not done (Supplementary Table 2, http://links.lww.com/MD/A584). Some representative examples are shown in Figures 3 and 4.

DISCUSSION

The poor prognostic impact of combined *MYC* and *BCL2* translocations ("DH") in DLBCL has been described in several studies.[3,7,33,34,43–45] Likewise, *TP53* mutation or deletion has been identified as poor prognostic factor in aggressive lymphomas.[8,9,42] However, there are only few reports which integrate all 3 markers[11,12,26,46] and data on the prognostic impact on cooccurrence of these aberrations are scarce. Here we have compiled a sufficiently large series of patients allowing for clinical outcome analysis. Our data show a complex interdependence of *TP53*+, *MYC*+, and *BCL2*+ with unexpected results.

Within our cohort *TP53*+ was the major outcome determinant, with a significant impact on OS as a sole parameter, and remaining significant in multivariate analysis. *TP53*+ were found in almost one-third of patients with equal distribution between the *MYC*+ and *MYC*− subgroups, as well as in the IHC assessed germinal center like (GCB) versus nongerminal center like (nGCB) groups. Clinical characteristics did not differ between *TP53*+ and *TP53*− patients at diagnosis. Importantly, more *TP53*+ patients were identified by subsequent sequencing analysis than by FISH only (19.8% vs. 11.9%). The frequency in our cohort was slightly higher than the reported 21% to 23% of *TP53*+ in unselected DLBCL.[8,9,47] Xu-Monette et al[42] reported a *TP53* mutation prevalence of 21.9% in de novo DLBCL and described *TP53* mutation as independent poor prognostic factor in the GCB subtype and, to a lesser extent, in the nGCB group. Gebauer et al[26] investigated the prevalence of *TP53* mutations in DHL and found an elevated frequency within *MYC*+/*BCL2*+ DHL with an incidence of 35.5% (6/17), compared to 6.25% (1/16) in *MYC*+/*BCL6*+ DHL. Information on clinical outcome was not provided. Hu et al found a similar incidence of *TP53* mutations in MYC/BCL2 protein positive and negative DLBCL. They observed a slight cumulative adverse impact on OS when MYC/BCL2 protein coexpression was combined with *TP53* mutation; however, these data were not significant and MYC and BCL2 status was assessed by IHC and not on a genetic level.[11] Another single institutional study based on IHC[37] reported a significant correlation of TP53 and MYC protein expression ($P = 0.001$) in a series of 85 DLBCLs. An enhanced negative impact on OS was observed when both proteins were overexpressed. Recently, the DHS based on IHC of the MYC and BCL2 proteins has shown significant discriminative power.[30] We also evaluated the DHS on our samples and found considerable overlap (data not shown). When patients with simultaneous expression of MYC and

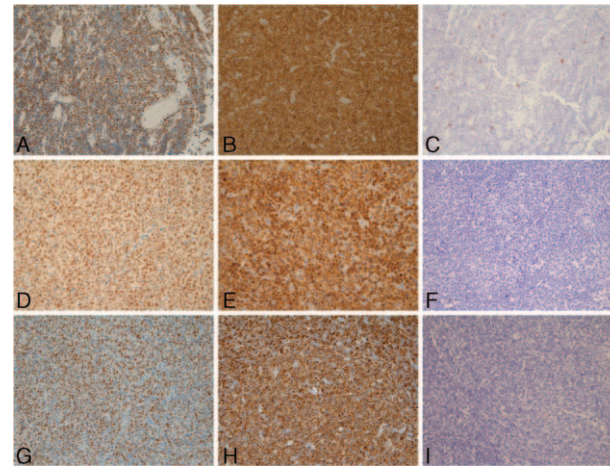

FIGURE 3. Immunohistochemistry staining profiles of exemplary double-hit patients. Three cases of MYC+/BCL2+ lymphomas. (A–C) Case #31; (D–F) case #37; (G–I) case #86. Panels A/D/G: positive staining for MYC protein; panels B/E/H: positive staining for BCL2; panels C/F/I: negative staining for TP53.

TABLE 3. Unadjusted and IPI Adjusted COX Regression Models of Overall Survival Comparing Single and Combined *BCL2* and *TP53* Aberrations in the *MYC* Positive Patient Group (N = 53)

	Unadjusted Model			Adjusted to IPI		
	P	**HR**	**95% CI**	***P***	**HR**	**95% CI**
BCL2+	0.18	2.188	0.690–6.936	0.48	1.531	0.465–5.041
TP53+	**0.01**	**4.338**	**1.339–14.057**	**0.002**	**7.006**	**2.035–24.124**
BCL2+*TP53*+	**0.04**	**0.119**	**0.016–0.871**	**0.03**	**0.097**	**0.013–0.757**

BCL2+, presence of *BCL2* translocation; *TP53*+, presence of *TP53* deletion/mutation; IPI, International Prognostic Index; *P*, *P*-value; HR, hazard ratio; 95% CI, 95% confidence interval.

Significant differences are indicated in bold letters.

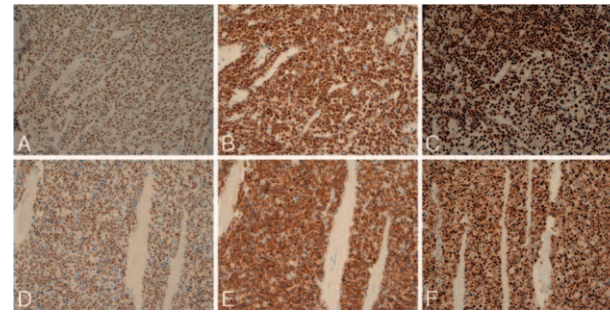

FIGURE 4. Immunohistochemistry staining profiles of exemplary triple-hit patients, Two cases of MYC+/BCL2+/TP53+ lymphomas. (A–C) Case #83; (D–F): case #26. Panels A and D: positive staining for MYC protein; panels B and E: positive staining for BCL2; panels C and F: positive staining for TP53.

BCL2 proteins were analyzed together with *TP53*+ a similar survival pattern as with genetic triple-hits was observed.

Sole *MYC*+ were associated with a particularly good outcome in our study. Patients with DHL had an inferior survival. A negative prognostic impact of *MYC* breaks in DLBCL, and particularly of the *MYC*+*/BCL2*+ DHL treated with both CHOP[48,49] and R-CHOP regimens[11,50–52] has been established in the literature. Despite this evidence the matter is not completely resolved. Some other reports[30,31,36] could not demonstrate a poor prognostic impact of a sole *MYC* translocation or MYC protein expression in DLBCL, but only when BCL2 (regardless of translocation or protein expression) was also altered (double-hit "DH").[30,31] In another study based on IHC[37] the authors have not detected significant differences in OS between MYC and BCL2 protein positive cases. Recently, Horn et al found a different biological risk profile in a young high-risk DLBCL cohort.[35] Breaks in the *BCL2* gene served here as the strongest negative prognostic marker in uni- and multivariate analysis for OS and PFS, whereas *MYC* break only showed a tendency toward inferior OS in these young patients.[35] The discrepancies in published results are probably due to the complexity of DLBCL biology[14,53] and the method of detection of *MYC*, *BCL2*, or *TP53* alterations at the protein or genetic level.

Most strikingly, *TP53*+ lost its negative influence in the presence of concurrent *BCL2*+ in our cohort. The combined aberration (*BCL2*+ in combination with *TP53*+) even improved survival. This interesting equalizing effect of *BCL2*+ in combination with a *TP53*+ was first observed by our group in an *Eμ-myc*-induced lymphoma mouse model.[38] In this genetic murine model disease latency was significantly influenced by the nature of the "second-hit," which was either defined as high expression of BCL2 or disruption of the TP53 pathway.[38] The latter led to disease progression through escape from immunosurveillance as shown in in vitro and in vivo experiments. Schuster et al[38] were able to ameliorate the negative effect of TP53-deficiency by enforced overexpression of BCL2, which resulted in reinduction of immunologic control mediated by functional NK and T cells. Although, this effect has potentially contributed to our results, there might be other explanations: In a healthy cell proapoptotic TP53 and antiapoptotic BCL2 are well-balanced counterparts. P53 may induce apoptosis by transcriptional activity through upregulation of proapoptotic BH3-only family members PUMA and NOXA and by nontranscriptional mechanisms through direct binding to BCL2 and its family proteins leading to BAX activation.[54] If this balance is destroyed by loss of *TP53* function through deletion/mutations or by overexpression of BCL2 protein, uncontrolled cell proliferation may occur as a consequence. Concurrent *TP53* and *BCL2* aberrations may thus render cells vulnerable and more sensitive for immunochemotherapy. However, we could not observe a higher Ki-67 proliferation index in this group of DLBCL patients (data not shown).

Generally accepted treatment recommendations for DLBCLs with *MYC* and *BCL2* translocations are still lacking. Since there is a consensus that DHL have an aggressive clinical course and poor response to conventional immunochemotherapy,[7,11,18,20,29,33,45,51] many patients with DHL have received high-dose methotrexate containing (HD-MTX) treatment. In our series, HD-MTX containing regimens were not superior to R-CHOP, particularly in patients over 60 years (data not shown). This is in line with previous results from the MRC/NCRI LY10[55] and data published by Snuderl et al.[28] Dose-adjusted EPOCH-R (etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, and rituximab) which has already been shown to be efficient in the treatment of adults with sporadic and immunodeficiency associated BL[56] and DLBCL with low and intermediate IPI,[57,58] produced also impressive remission in a small series of DHL.[59] Recently reported data indicate that BCL2 expression measured by IHC allows better prognostic discrimination than FISH.[59] It will be interesting to evaluate patients failing this therapy for additional genetic events, including *TP53*+. The findings from our study may also support the development of novel drugs, such as BH3 mimetics/BCL2 inhibitors.[60] A very recent study has evaluated the clinical effect of ibrutinib on DLBCL outcome. Interestingly, activated B-cell type lymphomas responded much better than GCB lymphomas.[61] It will be interesting to analyze the *TP53* status in this study.

Every retrospective study has some caveats. Patients were not uniformly treated but all patients received rituximab containing therapy. Our cohort was mainly collected in a referral center, possibly selecting for more advanced or aggressive cases. This might explain the somewhat higher number of *TP53*+ lymphomas. We did not exclude transformed follicular lymphoma (N = 8), thus possibly accounting for the accumulation of DHL. With the goal to focus on the most frequent genetic hits (*TP53*+, *MYC*+, and *BCL2*+) *BCL6* aberrations and *MYC* translocation partners were not assessed, since the inclusion of further factors would have caused the loss of statistic power in such a small sample size. Our patient group with concurrent *TP53*+ and *BCL2*+ is still small (7 *MYC*+*/BCL2*+*/TP53*+ and 2 *MYC-/BCL2*+*/TP53*+ patients). Nevertheless, this unique series of DLBCL patients with genetic work-up allowed important insights into the interdependence of genetic lesions as prognostic markers. The results force the necessity for comprehensive genetic and molecular diagnostics beyond the recognition of DH.

In conclusion, our data show that *TP53*+ occur in approximately one-third of all DLBCL and are associated with poor prognosis. However, subgroups with very different prognosis depending on the occurrence or co-occurrence of genomic aberrations of *TP53*, *MYC*, and/or *BCL2* can be defined. These findings provide further evidence for more extensive molecular or genetic diagnostics in aggressive lymphomas.

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