Prognostic impact of p53 aberrations for R-CHOP-treated patients with diffuse large B-cell lymphoma

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Abstract. Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoma in adults. There are specific alterations that appear repeatedly in DLBCL cases and play a role in lymphomagenesis or progression of the disease. Some aberrations were used as prognostic markers in the pre-rituximab era. Addition of rituximab to the classical anthracycline-based chemotherapy significantly increased the survival rate in DLBCL. Only few prognostic factors have been re-evaluated for patients treated with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone). We performed complex analysis of the *p53* tumor suppressor in collection of 75 DLBCL cases. Fifty-four patients were *de novo* cases, twenty-one cases developed into DLBCL by transformation from less aggressive disease. We determined functional status by analysis of separated alleles in yeast (FASAY) and analyzed the *p53* mutations by cDNA sequencing. We assessed the level of the p53 protein by immunoblot analysis. We used FISH to analyze loss of the *p53* and *ATM* (ataxia telangiectasia mutated) gene deletions. We detected 16 *p53* mutations (21.3%) including the mutation activating non-sense-mediated RNA decay pathway. Deletion of the *p53* allele was more common in cases with *p53* mutation. Mutations and/or deletions of *p53* had statistically significant negative impact on progression-free survival and tended to decrease also overall survival in 46 *de novo* DLBCL patients treated with R-CHOP. *p53* aberrations are negative predictors for survival of DLBCL patients treated with R-CHOP.

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Introduction

Diffuse large B cell lymphoma (DLBCL) is the most frequent lymphoma in adults. The tumor is characterized by centroblast-like and immunoblast-like cells expressing typical B-cell markers (CD19, CD20, CD22, CD79a) and the surface immunoglobulin (sIg). The DLBCLs vary in morphology, immunophenotype and clinical features (1,2). Gene expression profiling identified three distinct subgroups of DLBCL: germinal center B-cell-like (GCB), activated B-cell-like (ABC) and primary mediastinal (PM) DLBCL (3-7). The subgroups are derived from different cell of origin and arise by distinct genetic pathways (8). Several algorithms have been proposed to stratify DLBCL cases into GCB and non-GCB (ABC) subgroups according to the immunophenotype (9-12). These immunohistochemically defined subgroups differ in clinical outcome when treated with anthracycline-based chemotherapy (CHOP) (13,14), but this divergence is eliminated by addition of rituximab (14), chimeric monoclonal antibody binding to CD20 and activating apoptosis of CD20-positive cells (15). Addition of rituximab has significantly improved survival of DLBCL patients (16,17).

DLBCL is characterized by highly heterogeneous genetic and cytogenetic aberrations (18). Some alterations appear repeatedly in DLBCL cases probably playing a role in lymphomagenesis or progression of the disease. These include rearrangements involving *bcl-2*, *c-myc* (2) and *bcl-6* (19), *p16INK4A* inactivation and *p53* mutation (20). The adverse impact of *bcl-6* and *bcl-2*, was overcome by rituximab supplement (21,22).

Alterations of *p53* represent the most common genetic defect occurring in about half of all malignancies. In DLBCL, the *p53* mutations are detected in 13-22% of cases. Its association with worsened OS and PFS was described for DLBCL treated with chemotherapy alone (23-28).

p53 is a stress-responding tumor suppressor participating in control of the cell cycle, DNA repair, apoptosis and senescence (29,30). Inactivation of p53 increases cell proliferation and survival and promotes genomic instability and chemotherapy resistance of tumors (31). Inactivation of p53 usually results

from missense mutations in core region of the *p53* gene coding for the sequence-specific DNA-binding domain. The remaining *p53* allele can be inactivated by deletion, but some mutant p53 exert dominant-negative effect thus inactivating the wild-type p53 protein. The resulting mutated p53 protein might gain new oncogenic properties (32). Due to the inability to transactivate its negative regulator, the E3-ubiquitin ligase MDM2 (33-35), mutant p53 protein often accumulates in tumor cells.

Upstream of the p53 acts the serine/threonine kinase ATM responding to double-strand breaks in DNA (36). The p53 and ATM pathways overlap but they are not consistent in suppression of tumorigenesis. Combined status of both p53 and ATM could be an important determinant of the malignant phenotype (37,38). The *ATM* deletion shortened survival of patients with DLBCL (39). Other studies, however, did not find any association of the *ATM* deletion with prognosis of lymphoma patients (40-42).

We present results of complex analysis of the p53 tumor suppressor in 75 DLBCL cases. We determined the p53 status by FASAY and analyzed the *p53* mutations by cDNA sequencing. We assessed the p53 protein level by immunoblot analysis and used FISH to determine the loss of the *p53* gene. We used FISH also to study the *ATM* deletion. We statistically evaluated the role of *p53* aberrations in subset of *de novo* DLBCL patients treated with R-CHOP.

Materials and methods

Samples and patients. Seventy-five patients diagnosed with DLBCL (34 females and 41 males) underwent surgical biopsy of the tumor tissue in University Hospital in Brno from 2001 to 2009. Diagnosis of DLBCL was assessed by two experienced pathologists according to the WHO classification. Twenty-one patients developed DLBCL as a secondary tumor transformed either from follicular lymphoma (7 cases), chronic lymphocytic leukemia/small lymphocytic lymphoma (5 cases), marginal zone lymphoma (4 cases), low grade lymphoma not otherwise specified (3 cases) or nodular lymphocyte predominant Hodgkin lymphoma (2 cases).

For all patients, fresh-frozen tissue samples as well as formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks were available. Among primary DLBCL, in 12 cases, the analyzed material was obtained at the time of relapse. GC and non-GC subtypes were distinguished based on the algorithm of Hans *et al* (9). Clinical information was available for all cases. The characteristics of the patients with respect to sex, age, stage, International prognostic index (IPI) and treatment are listed in Table I. All patients provided informed consent.

FASAY and split assay. FASAY was performed as described earlier (43,44). Total RNA was purified using RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). cDNA was synthesized by SuperScript II (Life Technologies Inc., Carlsbad, CA, USA) using primer oligo$(dT)_{12}$. PCR was performed using primers P3 (5'-CCT-TGC-CGT-CCC-AAG-CAA-TGG-ATG-AT-3'), P4 (5'-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GT-3'), and *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). Yeast cells were co-transformed with the PCR product, linearized pSS16 plasmid, and the salmon sperm DNA carrier (Life Technologies) by the lithium acetate procedure (45).

Table I. Clinicopathological data of analyzed samples.

	De novo N=54 (%)	Transformations N=21 (%)	All N=75 (%)
Gender			
Male	31 (57.4)	10 (47.6)	41 (54.7)
Female	23 (42.6)	11 (52.4)	34 (45.3)
Age (years)			
Average	53	51	52
Median	57	54	54
Range	20-85	16-65	16-85
Distribution[a]			
GCB	20 (37.0)	11 (52.4)	31 (41.3)
Non-GCB	34 (63.0)	10 (47.6)	44 (58.7)
Stage			
I	4 (7.4)		4 (5.3)
II	14 (25.9)	1 (4.8)	15 (20.0)
III	12 (22.2)	3 (14.3)	15 (20.0)
IV	24 (44.4)	11 (52.4)	35 (46.7)
Unknown		6 (28.6)	6 (8.0)
IPI			
0	6 (11.1)		6 (8.0)
1	10 (18.5)	2 (9.5)	12 (16.0)
2	8 (14.8)	5 (23.8)	13 (17.3)
3	17 (31.5)	5 (23.8)	22 (29.3)
4	12 (22.2)	1 (4.8)	13 (17.3)
5	1 (1.9)		1 (1.3)
Unknown		8 (38.1)	8 (10.7)
IPI AA			
0	7 (13.0)		7 (9.3)
1	11 (20.4)	4 (19.0)	15 (20.0)
2	17 (31.5)	8 (38.1)	25 (33.3)
3	19 (35.2)	1 (4.8)	20 (26.7)
Unknown		8 (38.1)	8 (10.7)
ECOG			
0	16 (29.6)	3 (14.3)	19 (25.3)
1	15 (27.8)	9 (42.9)	24 (32.0)
2	16 (29.6)	1 (4.8)	17 (22.7)
3	6 (11.1)		6 (8.0)
4	1 (1.9)		1 (1.3)
Unknown		8 (38.1)	8 (10.7)
Primary therapy			
Standard	38 (70.4)	9 (42.9)	47 (62.7)
Intensive	14 (25.9)	2 (9.5)	16 (21.3)
No/unknown	2 (3.7)	10 (47.6)	12 (16.0)
PT response			
CR	47 (87.0)	12 (57.1)	59 (78.7)
PR	2 (3.7)	1 (4.8)	3 (4.0)
Prog	2 (3.7)	1 (4.8)	3 (4.0)
Unknown	3 (5.6)	7 (33.3)	10 (13.3)

[a]According to Hans *et al* (9).

Transformed yeast cells were plated on minimal medium lacking leucine and with 5 μg/ml of adenine, followed by

incubation at 35˚C for 2-3 days, and then for 2-3 days at room temperature. For split assay, PCR of the *p53* 5'-part was performed with primers P3 and P17 (5'-GCC-GCC-CAT-GCA-GGA-ACT-GTTACA-CAT-3'), the 3'-part with primers P4 and P16 (5'-GCGATG-GTC-TGG-CCC-CTC-CTC-AGC-ATC-TTA-3'). Yeast cells were transformed with linearized vectors pFW35 and pFW34 (46).

FASAY deduces the functional status of p53 from color of colonies of transformed yeast cells. Expression of functional p53 results in large white colonies, inactive p53 leads to smaller red colonies. The background frequency of red yeast colonies typically does not exceed 10%. Thus, samples providing <10% of red colonies are considered to contain only wild-type *p53* alleles, while samples providing >10% of red colonies are suspicious to bear a clonal *p53* mutation (43). The ratio of red colonies scoring between 10 and 20% can result from a presence of clonal *p53* mutation in rather small portion of cells or from increased degradation of RNA. To distinguish these two possibilities, version of FASAY, called split assay, was established. In the split assay, the 5'- and 3' parts of the *p53* cDNA are tested separately (46).

Purification of the plasmids from transformed yeast cells and sequencing of the p53 cDNA. Yeast cells from individual yeast colonies were harvested, resuspended in TSN (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), and grinded by vortexing with glass beads; plasmid DNA was extracted by phenol/chloroform procedure. The *p53* cDNA was amplified using the P3 and P4 primers and Taq polymerase (Life Technologies) and subjected to agarose gel electrophoresis. The PCR product was purified by MinElute PCR purification kit (Qiagen) and sequenced by BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Isolation of gDNA and amplification of exons 6 and 8 of p53. Genomic DNA was isolated from formalin-fixed, paraffin-embedded tissue blocks using the purogene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Exons 6 and 8 of the p53 gene were amplified by PCR using primers p53-Pg6Fa (5'-AGAGACGACAGGGCTGGTT-3'), p53-Pg6R (5'-CTTAACCCCTCCTCCCAGAG-3', p53-Pg8Fb (5'-GCC-TCT-TGC-TTC-TCT-TTT-TCC-3'), p53-Pg8R (5'-TAA-CTG-CAC-CCT-TGG-TCT-CC-3') and Taq polymerase (Life Technologies). The PCR products were purified and sequenced as described above.

FISH. FISH was performed on tissue sections prepared from FFPE blocks. For the *p53*-specific locus analysis, the Vysis LSI TP53 (17p13.1) Spectrum Orange probe and the centromeric CEP 17 Spectrum Green DNA probe were used. For analysis of *ATM* locus, Vysis LSI ATM (11q22.3) Spectrum Orange probe and CEP 11 Alpha Spectrum Green DNA probe were used (Abbott Molecular Inc., Abbott Park, IL, USA). Hybridization was performed according to the manufacturer's instructions. Images were scanned by Leica DMRXA2 microscope equipped with CCD camera (COHU). Fluorescence signals were analyzed using Leica Q-FISH software (Leica Microsystems GmbH, Wetzlar, Germany). Cells (50-100) per case were analyzed. The cut-off level was defined by the mean value plus three times the SD of the frequency of control cells exhibiting one red and two green signals (9.6% for p53 and 9.8% for *ATM*).

Array comparative genomic hybridization (CGH). Whole-genome analysis of chromosomal changes in case 44 was performed using oligonucleotide Human Genome CGH microarray 44K (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Briefly, 1 μg of reference DNA (Promega Corp., Fitchburg, WI, USA) and test DNA were enzymatically restricted and labeled (Cy3-dUTP and Cy5-dUTP) using the random-priming reaction. Purified (Microcon YM-30 filters, Millipore), differentially labeled test and reference DNA were co-hybridized to the array containing about 43.000 short (60-mer) oligonucleotides representing both coding and non-coding sequences of whole human genome. After one day of hybridization, and washing off the unhybridized probes, microarrays were scanned with Agilent Microarray Scanner. Data were obtained using Feature Extraction software (v. 6.1.1) and visualized by CGH Analytics software (v. 3.5.14) (Agilent Technologies). Copy number changes were detected using ADM-2 algorithm with ≥3 neighbouring oligos with significantly aberrant intensity ratios patient/reference.

Immunoblotting. Tissue samples were lysed in 150 mM NaCl, 50 mM NaF, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% NP40 and 1 mM phenylmethylsulfonyl fluoride in ice for 30 min, and the cell extract was centrifuged at 17,000 x g for 30 min to remove cell debris. Protein concentration was measured by the Bradford assay. Solubilized proteins were resolved by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked in 0.1% Tween-20 and 5% low-fat milk in PBS for 1 h and probed with anti-p53 mouse monoclonal antibody DO-1 (kindly provided by B. Vojtesek) at 4˚C. Blots were developed with Dako peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark) using the ECL chemiluminescence detection kit (Amersham Biosciences, Vienna, Austria).

Statistical analyses. Frequency tables and descriptive statistics (mean, median, minimum and maximum) were used for summarizing characteristics of the patients. Differences between compared groups of patients were assessed by Maximum Likelihood Chi-square test and Fisher exact test in categorial variables and by Mann-Whitney test in continuous variables. Graphic visualization of patients' survival according to monitored parameters was performed using Kaplan-Meier survival curves. Differences in survival were tested using the log-rank test. As a level of statistical significance α=0.05 was used. Analysis and graphic visualization were performed in statistical software SPSS 12.0.1 for Windows and Statistica 8.0 for Windows.

Results

Assessment of the p53 status by FASAY. We performed FASAY of all 75 samples (Table II). Fifty-eight cases scored under the background 10% level, 15 cases were positive and scored

Table II. The cases with a p53 aberration.

Case	FASAY%[a]	*p53* mutation	FISH %[b]	WB (DO-1)
De novo DLBCL				
5	21.7	G244S	0.0	-
8	12.1	R196stop	0.0	-
51	80.3	R248G	0.0	-
18	30.0	R273H	0.0	+
67	62.4	del10nt 276-279	1.9	+[c]
7	96.8	G245D	0.0	+++
73	74.1	R273H	1.8	+++
47	46.3	R273C	0.0	+++
35	32.0	L257R	4.7	-
28	78.0	C135R	38.5	+
30	73.6	I254N	29.5	+
50	74.5	R175H	53.6	+++
23	1.3	-	0.0	+++
60	5.0	-	0.0	+++
2	5.3	-	18.8	-
43	ND	ND	29.5	-
Transformations				
3	70.0	C135G	63.7	+++
4	94.0	del 18nt 177-182	49.1	+++
44	83.4	V272G	50.0	+++
40	60.0	C238F	0.0	+++
53	7.5	-	50.8	-
61	3.5	-	84.3	+

[a]% of red yeast colonies; [b]% of cells with *p53* deletion; [c]truncated protein.

above 20% of red colonies (ranged from 21.7 to 96.8%). In case 8, the result of FASAY was ambiguous as it scored 12.1%. The split assay provided a rather symmetric result (11.6% of red colonies for the 3'-part and 19.8% for the 5'-part of the *p53* cDNA). From our previous analyses we know that this kind of result does not exclude the presence of a clonal *p53* mutation. Therefore, this case was considered positive and further analyzed by cDNA and gDNA sequencing. We were not able to explicitly analyze case 43, we repeatedly obtained only faint product of the *p53* PCR and highly fluctuating results of FASAY.

Sequencing of the p53 cDNA. The 16 cases positive in FASAY/split assay were analyzed further by cDNA sequencing. The p53 expression vector was recovered from 3 to 6 red colonies per case and the isolated cDNA was used as a template for sequencing. In all 16 cases, we detected clonal *p53* mutations, i.e. the mutations were found in majority of analyzed colonies (Tables II and III). All detected *p53* mutations were localized to the DNA-binding domain. Thirteen mutations (81.3%) were missense. The non-sense mutation in case 8 formed premature termination codon in position 196. Mutations detected in cases 4 and 67 were recognized as deletions of 12 and 10 nucleotides, respectively. The deletion in case 67 resulted in the reading frame shift and formation of premature termination codon in position 345. We isolated gDNA from FFPE tissue of case 8 and sequenced exon 6 of the p53 gene (Fig. 1). We confirmed the presence of mutation R196 stop in the gDNA. The mutated *p53* allele represented majority in the sample contrary to results of FASAY showing presence of mutation in only rare colonies. This strongly suggests that the premature stop codon might activate the nonsense-mediated RNA decay pathway (47,48).

Overall, a clonal *p53* mutation was detected in 16 DLBCL cases (21.3%), 12 of them in *de novo* DLBCL cases. Two mutations (cases 35 and 47) were found in the tissue samples from disease relapse. We confirmed mutation R273C by sequencing of gDNA (exon 8) isolated from the primary biopsy FFPE block of case 47. In case 35, the quality of isolated gDNA was insufficient for DNA amplification.

The distribution of the detected p53 mutations was non-random. Six out of 13 missense mutations (46%) affected 4 hot-spot codons (R175, G245, R248, R273), all 13 mutations spanned into evolutionary conserved regions, 6 were in region IV, 4 in region V, 2 in region II and 1 in region III. Six mutations were in exon 7, 4 in exon 8 and 3 in exon 5.

Table III. Results of the p53 and ATM analyses.

	De novo N=54 (%)	Transformations N=21 (%)	All N=75 (%)
p53 mutation			
Yes	12 (22.2)	4 (19.0)	16 (21.3)
No	42 (77.8)	17 (81.0)	59 (78.7)
p53 deletion			
Yes	5 (9.3)	5 (23.8)	10 (13.3)
No	49 (90.7)	16 (76.2)	65 (86.7)
p53 mutation and/or deletion			
Yes	14 (25.9)	6 (28.6)	20 (26.7)
No	40 (74.1)	15 (71.4)	55 (73.3)
p53 protein (WB)			
-	28 (51.9)	12 (57.1)	40 (53.3)
+	16 (29.6)	3 (14.3)	19 (25.3)
++	4 (7.4)	2 (9.5)	6 (8.0)
+++	6 (11.1)	4 (19.0)	10 (13.3)
ATM deletion			
Yes	6 (11.1)	4 (19.0)	10 (13.3)
No	45 (83.3)	17 (81.0)	62 (82.7)
ND	3 (5.6)		3 (4.0)

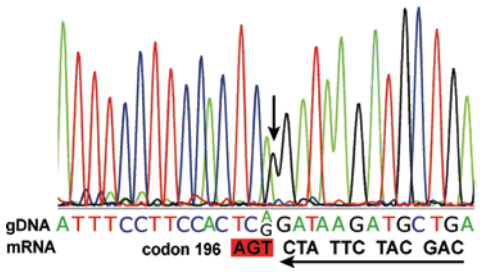

Figure 1. Result of the *p53* gDNA sequencing of case 8. Arrow shows G to A substitution in the position 196 resulting in formation of premature termination codon.

Mutations C135G, I254N and V272G were temperature sensitive (49).

Detection of the 17p13.1 and 11q22.3 loci. We performed FISH analysis of both *p53* and *ATM* alleles using the locus-specific (red) and centromeric (green) probes. In some cases, we found presence of only one red and one green signal per nucleus (1R+1G) in considerable high proportion of cells (Fig. 2). In case 44, for instance, we detected 1R+1G pattern in 46.0% of nuclei when analyzing *p53* and in 64.5% of nuclei when assessing *ATM*. To get deeper insight into the chromosome status in this case, we performed array-CGH analysis. We found long deletion overlapping centromeric region on both chromosome 17 (del 17p13.3-q21.2) and 11 (del 11p11.2-q25) (Fig. 2). This result clearly explained our observation by FISH. Nuclei containing one red and one green signal were considered as positive for *p53/ATM* loss. Altogether, deletion of the *p53* allele was found in 10 cases (Tables II and III). In six cases

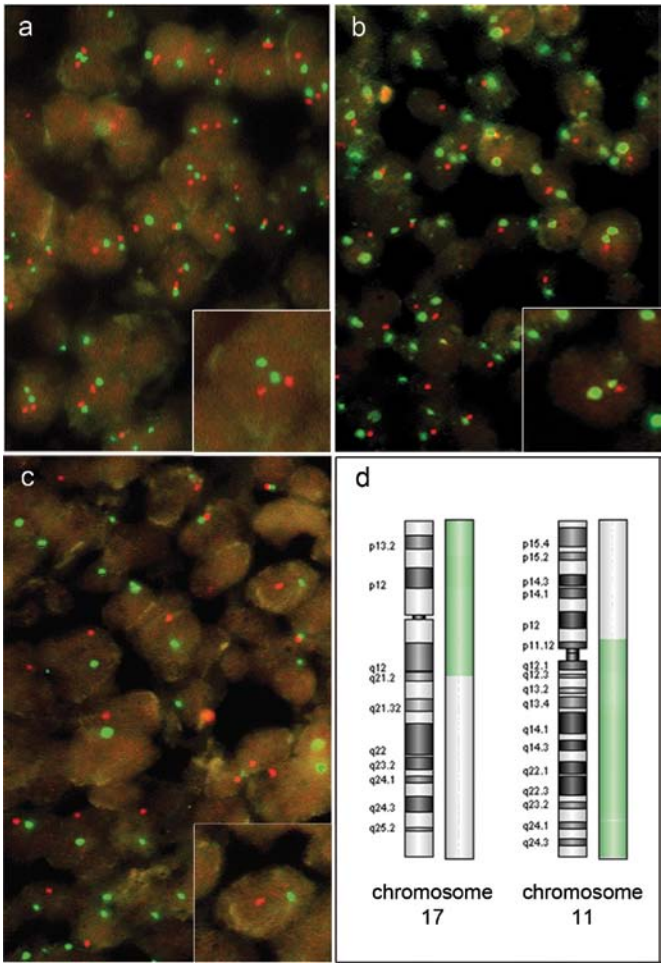

Figure 2. Detection of the *p53* and *ATM* deletion by FISH and array CGH. (a) nuclei negative for deletion of *p53* (2R+2G); (b) nuclei positive for deletion of *p53* (2G+1R); (c) nuclei positive for deletion of p53 (1G+1R); (d) schematic representation of array CGH in case 44, the parts of chromosomes highlighted in green were deleted.

the remaining *p53* allele was mutated. Deletion of *ATM* allele was also detected in 10 cases (Table III), whereas concurrent deletion of both *p53* and *ATM* was found in 3 samples (cases 30, 44, 61).

Analysis of the p53 protein by immunoblotting. The level of p53 was assessed according to the control; human breast cancer cell line BT474. The BT474 cells bear *p53* mutation E285K and constitutively overexpress the p53 protein (+++). We found 10 DLBCL cases with high level of the p53 protein (+++), 8 of them having the *p53* gene mutated. Six cases featured median level (++) and 19 cases low level (+) of the p53 protein, three of them had *p53* affected by missense mutation. In 40 cases, including 3 cases bearing *p53* missense mutation, no p53 protein (-) was detected (Fig. 3).

The relationship between the p53 status and the disease outcome. We compared selected parameters separately in *de novo* DLBCL cases and in patients with transformed DLBCL. These two groups differed in some clinical parameters, such as disease stages (p<0.001), IPI AA categories (p=0.026), ECOG categories (p=0.041), rate of complete remission (p=0.01), e.g. as

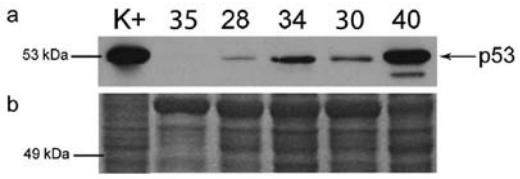

Figure 3. Assessment of the p53 protein level by immunoblotting using DO-1 antibody (a) and total protein fraction stained with coomassie brilliant blue (b). K^+, positive control; human cell line BT474 bearing mutation E285K.

shown in Table I. The distribution of detected p53 aberrations between the two groups is summarized in Table III. Median overall survival (OS) for the whole cohort was not reached. Median progression-free survival (PFS) was 59.9 months. We did not find any statistically significant difference in survival (both OS and PFS) between *de novo* and transformed DLBCL groups of patients.

The overall survival of *de novo* DLBCL patients (N=54) was not affected by any p53 aberration (*p53* mutation, *p53* allele deletion, *p53* mutation and/or deletion, p53 protein overexpression). The presence of *p53* mutation and/or deletion decreased PFS in this group of patients but the result was on the border of statistical significance (p=0.051). However, the evaluated group of *de novo* DLBCL patients was heterogeneous in terms of applied therapy. Eight cases did not receive rituximab compared to 46 *de novo* DLBCL patients who were treated with R-CHOP therapy. All 12 cases with mutated *p53* were treated with R-CHOP, in 3 of them the mutation was accompanied by deletion of the second allele. In case 43 with deletion of *p53* allele the mutational status was not evaluated. Together there were 13 patients with mutation and/or deletion of *p53* among cases treated with R-CHOP. The statistical analyses of survival in those 46 patients revealed a clear difference in OS as well as PFS in dependence on *p53* mutation and/or deletion. For PFS the result was statistically significant (p=0.021), 72.1% of patients without any *p53* aberration and only 33.3% of patients with mutations and/or deletions of *p53* survived 24 months (Fig. 4).

The group of 21 transformed DLBCL cases was too small to provide statistically significant results but the presence of *p53* mutation and/or deletion also decreased OS as well as PFS of patients. Interestingly, *de novo* DLBCL patients with deletion of *ATM* (N=6) exhibited better survival rate (both OS and PFS) compared to patients with both *ATM* alleles (N=45). Due to low number of cases with loss of *ATM*, the results were not statistically significant.

Discussion

We detected mutation of the *p53* gene in 16 (21.3%) cases. Twelve of them were found in *de novo* DLBCL patients (22.2%), four in transformed cases (19.0%). The mutational rate in our study is in good agreement with other studies where the frequency of the *p53* mutations ranged mostly from 13 to 23% (23-25,27,28,50-53) as well as with multicentric study comprising 477 DLBCL patients, in which the mutational ratio was 21.4% (26).

In our previous study on mantle cell lymphomas, we identified deletion of the *p53* allele only in cases with mutated *p53* and also the p53 protein overexpression was associated only with *p53* mutations (48). In contrast, in this study patients lacking the *p53* mutation with loss of *p53* allele and/or p53 protein overexpression were found. The p53 protein was mainly accumulated in tissues containing mutant *p53* (80% of cases having high protein expression contained *p53* mutation). High p53 protein level in cases with functional p53 was described also by Young *et al* (25) and Villuendas *et al* (54). These results suggest that besides the *p53* mutations there are other mechanisms leading to stabilization of p53 in DLBCL cancer cells and highlight necessity to analyze both mutational status of the *p53* gene and the p53 protein level.

The *p53* mutations were described to decline the rate of complete remission in DLBCL patients (23,25). Several studies showed significant impact of the *p53* mutations on OS of DLBCL patients (23,25,26,28). However, there are other studies with contrary findings on adverse effect of the *p53* mutations on prognosis of NHL patients (52,55). According

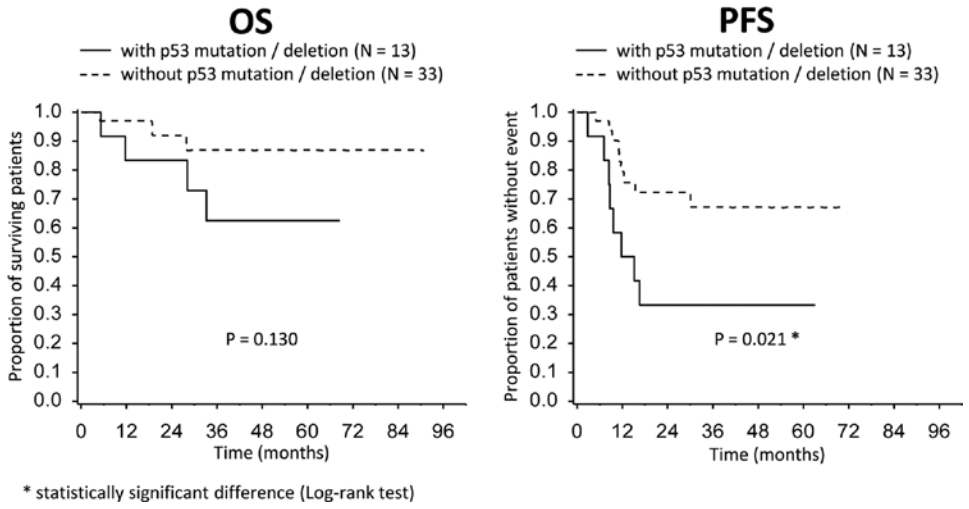

Figure 4. Overall survival (OS) and progression-free survival (PFS) of patients with *de novo* DLBCL treated with rituximab according to presence of *p53* mutation and/or *p53* deletion.

to the collaborative study of Young *et al* (25), the *p53* deletion has no adverse impact on DLBCL patients survival while the level of mutant p53 protein negatively influences the survival rate of patients.

In our present study, we prove statistically significant adverse effect of the *p53* aberrations on PFS of DLBCL patients treated with R-CHOP. The tendency of *p53* aberrations to decrease OS is also clearly shown. The main reason why the statistically significant level was not reach is probably the small number of patients in the study. Other studies analyzed larger groups of DLBCL patients [102 (23); 113 (25); 477 (26); 102 (27)]. We did not observe a negative effect of high p53 protein level on *de novo* DLBCL patient survival. Some samples accumulating p53 were not affected by *p53* mutation suggesting different mechanism responsible for the p53 protein stabilization. High level of functional p53 could provide different effect than high level of mutated p53.

The *ATM* gene is believed to play an important role in lymphomagenesis and the deletion of this locus was shown to correlate with lymphoma phenotype (39). Loss of the *ATM* allele was supposed as a second hit after mutation in the second allele (41). However, cases with deletion of one allele without any mutation also exist (40). We found *ATM* deletion in 13.9% of analyzed cases and in 11.1% of *de novo* group similarly to Cuneo *et al* (39). The *p53* and *ATM* mutations occur independently in MCL (41), while in DLBCL non-random coexistence of these alterations was found (37). Simultaneous inactivation of both p53 and ATM resulted in excellent prognosis in the study of Jiang *et al* (38). In our study, 7 cases with *ATM* deletion did not have any aberration of *p53*. Three *ATM* deletion-positive cases had *p53* deletion two of them carry *p53* temperature-sensitive mutation in the second allele. Two of these patients (one with and one without p53 mutation) responded well to initial treatment and are in complete remission while the remaining case (44) transformed from follicular lymhoma and died 5 months later.

In conclusion, we performed complex analysis of the p53 aberrations in DLBCL. We used combination of several alternative methods to address the p53 function and showed that this complex approach provides valuable insight into the ways of the p53 impairment. Although the group of R-CHOP-treated DLBCL patients analyzed in this study was rather small, we showed that the *p53* aberrations retain adverse effect on their survival.

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