TP53 DNA binding domain mutational status and rituximab-based treatment are independent prognostic factors for pediatric Burkitt lymphoma patients stratification

Although current chemotherapy regimens are extremely effective in curing Burkitt lymphoma (BL) in children, the outcome for patients with primary refractory or relapsed disease still remains very poor, with fewer than 30% successfully salvaged despite the use of high-dose chemotherapy and stem cell transplant.[1] Moreover, the high success rates of first-line chemotherapy, mainly due to the addition of anti CD20 to standard chemotherapy,[2-4] are reached at the cost of significant acute toxicity and long-term sequelae.[5,6] In this scenario, due to the lack of consistent data regarding effective salvage regimens for relapsed/refractory disease, the early identification of patients at high risk of treatment failure is mandatory to properly refine treatment. In the present study, we performed *TP53* DNA binding domain (DBD) mutational analysis on a very large pediatric BL cohort. By multivariate analysis, we demonstrated the independent prognostic impact of *TP53* mutational status for the early identification of BL patients at higher risk of treatment failure.

For more than ten years, minimal disseminated disease (MDD) and minimal residual disease (MRD) have been the only biological criteria that allow patients with increased risk of relapse/resistance to be identified, as demonstrated by our studies on large cohorts of BL patients enrolled in the AIEOP LNH-97 treatment protocol.[7] More recently, starting from the observation that the presence of *TP53* mutations is significantly associated with adverse outcomes in adult aggressive B-cell lymphomas, 2 large independent studies used univariate analysis to demonstrate that *TP53* abnormalities define clinical risk groups also in pediatric BL.[8,9]

The present retrospective study includes a cohort of 214 pediatric BL patients enrolled in Italy between January 1999 and February 2022. The inclusion criteria were the availability of both the tumor tissue and bone marrow (BM) and/or peripheral blood (PB) at diagnosis to perform *TP53* mutational status and MDD analyses. For 142/214 patients, BM/PB before the second chemotherapy cycle was also available to perform MRD analyses. Patients were treated according to the AIEOP LNH-97 (N=196)[5] or the Inter B-NHL 2010[4] (N=18) treatment protocols. Overall, 66 out of 214 patients received anti-CD20 monoclonal antibody (rituximab) injections in addition to chemotherapy. The main clinical characteristics of the study population are reported in Table 1. The diagnosis of BL was established from clinical, histological, and immunohistochemistry findings. In all cases, the histological diagnosis was centrally reviewed. The study was approved by the ethics committees of each participating institution and the informed consent of the parents or legal guardians was obtained before patients' enrollment. Epstein-Barr virus (EBV) detection and copy number evaluation was assessed by performing real-time polymerase chain reaction on DNA samples from all cases. DNA from an EBV$^+$ Namalwa BL cell line was used to produce a calibration curve with known EBV copy numbers (Namalwa harbors 2 integrated EBV copies/cell).[10] Among our cohort, only 9% of patients were EBV$^+$.

TP53 gene hot-spot exons 5, 6, 7 and 8 were amplified according to the IARC protocol (https://tp53.isb-cgc.org/pdf/TP53_SangerSequencing_IARC). Primer sequences were as follows: exon 5: 5'-TTCACTTGTGCCCTGACTTTCA-3', 5'-CAGCCCTGTCGTCTCTCCAG-3'; exon 6: 5'-GCCTCTGATTCCTCACTGAT-3', 5'- TTAACCCCTCCTCCCAGAGA-3'; exon 7: 5'-CTTGCCACAGGTCTCCCCAA-3', 5'- AGGGGTCAGAGGCAAGCAGA-3'; exon 8: 5'-TTCCTTACTGCCTCTTGCTT-3', 5'- AGGCATAACTGCACCCTTGG-3'. PCR amplicons were purified using the Illustra ExoProStar 1-Step reagent (Cytiva, Marlborough, MA, USA) and sequenced on a 3500 DX Genetic Analyzer (ThermoFisher Scientific, Waltham, MA, USA). Electropherograms were visually inspected by Sequence Scanner Software v2.0 (Applied Biosystems) and sequences compared to NM_000546.6 as the reference sequence. *TP53* copy number values were determined for 175/214 by using the SALSA MLPA probemix P056-D1 (MRC-Holland) and results analyzed with Coffalyser software v.220513.1739 (MRC-Holland). The combination of Sanger sequencing and MLPA analyses allowed us to discriminate between monoallelic/biallelic genomic lesions. Overall, 98 cases were defined wild-type, whereas 48 and 15 cases showed monoallelic or biallelic genomic lesions, respectively. Of the 48 cases with monoallelic abnormalities, 43 had a single somatic heterozygous mutation and 5 had copy number variations (CNV). As for the 15 cases with biallelic abnormalities, 4 had a mutation combined with a heterozygous deletion of the entire gene and 11 displayed a homozygous mutation. For the 14/175 remaining cases, including 4 cases with both a CNV and a mutation, and 10 cases with multiple mutations, the combination of Sanger sequencing and MLPA analyses did not allow us to distinguish between monoallelic/biallelic genomic lesions.

In line with previous studies conducted by the UK (57.8%)[8] and German (52.9%)[9] groups, *TP53* mutations were detected in 87/214 (40.7%) of our cases. The lower prevalence of mutations in our cohort could be attributed to the different sequencing approaches, since Newman[8] and Burkhardt[9]

analyzed the complete *TP53* coding sequence by whole-exome and targeted deep sequencing, respectively, while we focused on the DNA binding domain (DBD) by performing Sanger sequencing of hot-spot exons 5 to 8. In line with the vast majority of tumors and BL cases bearing *TP53* mutations, also in our cohort, mutations in *TP53* were mostly represented by missense mutations.[11] Indeed, of the 102 identified mutations, 93 were missense, 5 were nonsense, 2 were in-frame deletions, one was a frameshift mutation, and one involved a splicing donor site (Figure 1). Moreover, 13/87 patients showed 2 co-existing mutations, 1/87 showed 3 and 17/87 showed only the mutated allele (*Online Supplementary Table S1*). The mutation frequency of *TP53* DBD coding exons ranged from 8% (exon 6) to 38% (exon 7), with exon 5 and 8 mutated in 30% and 24% of the patients, respectively (Figure 1). The most frequently detected variants were the hot-spot mutations R175H, detected in 11/30 patients with mutated exon 5, and the R248Q and R248W, detected in 17/38 and 8/38 patients with mutated exon 7, respectively. The hotspot residue R273 in exon 8 was affected in 8/24 patients with mutated exon 8 (R273C in 3 cases, R273H in 5 cases).

Overall, the presence of *TP53* mutations was associated with a significantly inferior outcome (Figure 2A, *Online Supplementary Figure S1A*). In line with the trend observed by Newman,[8] the presence of biallelic abnormalities were associated with a significantly inferior PFS compared to monoallelic genomic alterations (Figure 2B, *Online Supplementary Figure S1B*).

As for the most frequently detected variants, the R175H mutation had the same prognostic impact of other mutations affecting exon 5 (Figure 1C, *Online Supplementary Figure S1C*), whereas the R248Q substitution was significantly associated with a better outcome: patients bearing the R248Q substitution showed a progression-free survival (PFS) and overall survival (OS) very similar to patients

Table 1. 3-year progression-free survival univariate and multivariate analysis based on clinical and biological characteristics of 214 pediatric Burkitt lymphoma patients.

Patients' characteristics	Patients, N	Events, N	3-year PFS % (SE%)	Univariate *P*	Multivariate *P*	Hazard Ratio (95% CI)
Gender						
Male	180	24	86 (3)	0.088	NS	-
Female	34	8	76 (7)			
Median age in years						
<7.7	111	14	87 (3)	0.293	NS	-
>7.7	103	18	85 (3)			
BM involvement*						
No	188	27	80 (8)	0.518	-	-
Yes	26	5	77 (10)			
CNS involvement						
No	200	30	84 (3)	0.968	-	-
Yes	14	2	85 (10)			
Risk group#						
1-2-3 and B	45	1	98 (2)	0.01	NS	-
4 and C	166	30	81 (3)			
Stage°						
1-2	38	1	97 (3)	0.0259	NS	-
3-4	176	31	82 (3)			
MDD						
Neg	122	14	88 (3)	0.109	NS	-
Pos	92	18	80 (4)			
Rituximab						
No	147	26	82 (3)	0.092	0.0318	0.4 (0.1-0.9)
Yes	66	6	90 (4)			
TP53						
WT	127	12	90 (3)	0.0055	0.0247	2.3 (1.1-4.9)
Mut	87	20	77 (5)			

BM: bone marrow; CI: Confidence Interval; CNS: central nervous system; MDD: minimal disseminated disease; PFS: progression-free survival (defined as the time elapsed between date of diagnosis to the date of the first event [relapse, refractory disease, disease progression] or to the date of the last follow up); SE: standard error; WT: wild-type; Mut: mutated; NS: not significant. *BM involvement was defined on the basis of smear morphological examination. °St Jude staging system.[15] #Risk group 1-4 was defined according to the treatment protocols of the patients, i.e., AIEOP LNH97[5] and risk group B-C according to Inter B-NHL Ritux 2010.[4]

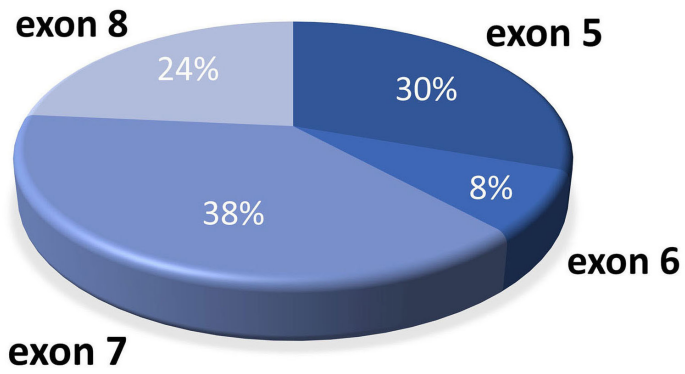

Figure 1. Mutational overview of *TP53* DNA binding domain. (A) Mutation frequency observed in each exon encoding for *TP53* DNA binding domain. (B) Lollipop plot representing 102 mutations identified in 87 Burkitt lymphoma (BL) patients and their classification. SNV: single nucleotide variant.

with wild-type *TP53* as compared to patients bearing the R248W or other mutations affecting different residues in exon 7 (Figure 1D, *Online Supplementary Figure S1D*). Only 3/19 EBV⁺ cases showed *TP53* mutation; all EBV⁺ patients obtained a continuous clinical remission.

It is noteworthy that, when clinical factors were also considered, *TP53* status and rituximab administration were the only prognostic factors in multivariate analysis (Table 1). These results further suggest that *TP53* mutational status is the most promising biological risk factor for BL patient risk-based stratification and that rituximab is effective in improving the cure rates of this aggressive pediatric lymphoma.[8]

Minimal disseminated disease at diagnosis and MRD after the first chemotherapy cycle were performed in BM and/or PB from each patient analyzing the presence of t(8;14)

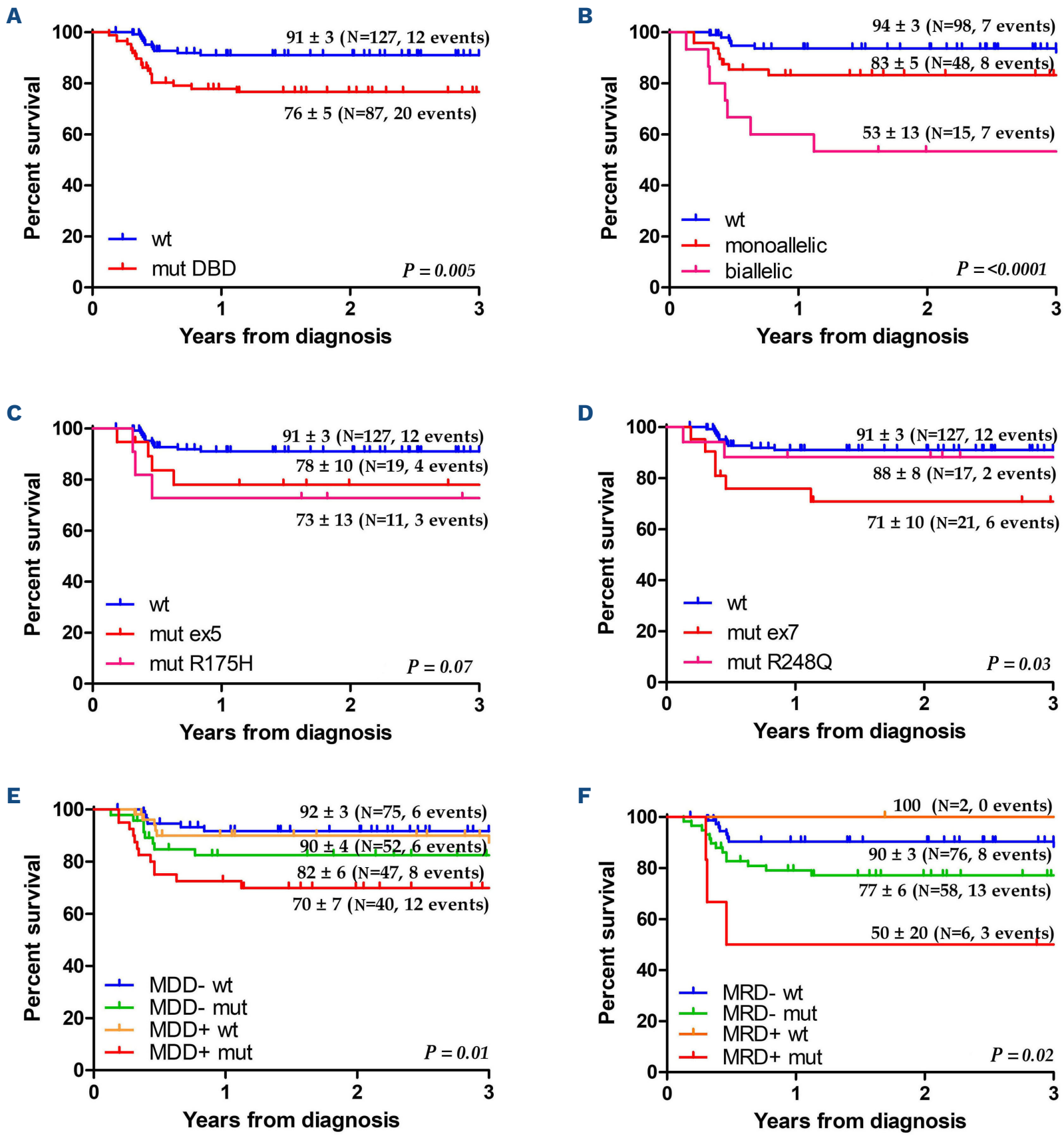

Figure 2. Progression-free survival according to *TP53* mutational status, alone and in combination with disease dissemination. 3-year progression-free survival % according to (A) *TP53* DNA binding domain (DBD) mutational status, (B) the presence of biallelic/monoallelic mutations on DBD, or (C and D) the presence of specific hot-spot mutations in exons 5 and 7. (E and F) Combined significance of *TP53* mutations and minimal disseminated disease (MDD) or minimal residual disease (MRD), respectively. mut: mutation; N: number; wt: wild-type.

(q24;q32) by long-distance PCR (LD-PCR), as previously published.[5,12,13] For 48 cases with t(8;14) negative tumor biopsy, MDD/MRD analysis was performed by immunoglobulin rearrangements.[14]

This analysis did not show prognostic significance. This seems in contrast to our previous data, but the present study cohort included, for the first time, patients who received rituximab (N=66). Indeed, when we focused on BL patients who did not receive rituximab, MDD$^+$ patients showed a significantly inferior outcome compared to MDD$^-$ patients (*Online Supplementary Figure S2A, B*), whereas patients who received rituximab displayed similar PFS both in the presence or absence of MDD (*Online Supplementary Figure S2C, D*), suggesting that rituximab addition to chemotherapy might overcome the prognostic impact of molecular disease dissemination.

However, in order to understand if MDD might improve risk stratification in *TP53* mutated patients, we analyzed the combination of these 2 parameters and we identified a group of BL patients at very high risk of treatment failure (P=0.01) (Figure 2E, *Online Supplementary Figure S2E*). Indeed, patients both MDD$^+$ and with mutated *TP53* showed a 3-year PFS of 70% ± 7% compared to 82% ± 6% for patients bearing *TP53* mutations without disease dissemination. Furthermore, the combination of *TP53* mutations and MRD identified a small group of patients at an even higher risk of treatment failure (P=0.02) (Figure 2F, *Online Supplementary Figure S2F*). Among them, all patients performed a uniform 6 chemotherapy courses, except one who had additional anti-CD20. Three out 6 patients experienced progressive disease and died. No association to clinical characteristics were observed.

In conclusion, our results on the independent prognostic impact of *TP53* mutations on pediatric BL response to treatment further confirm the importance of this biological parameter for the early identification of BL patients at increased risk of treatment failure. MDD positivity should be considered a warning to define later the very high-risk patients who should be candidates for innovative therapeutic strategies. Overall, the results of our study will significantly contribute to the design of a new risk-based international treatment protocol for pediatric BL and to the definition of the biological risk factors to be assessed in the treatment decision-making process.

Authors

Gaia Martire,[1,2*] Federica Lovisa,[1*] Elisa Carraro,[2] Domenico Rizzato,[1,2] Simone Cesaro,[3] Rosa Maria Mura,[4] Annalisa Tondo,[5] Cinzia Bertolin,[1,6] Francesca Boaretto,[1,6] Leonardo Salviati,[1,6] Alessandra Biffi,[1,2] Marta Pillon[2] and Lara Mussolin[1,2]

[1]Istituto di Ricerca Pediatrica Città della Speranza, Padova; [2]Paediatric Hematology, Oncology and Stem Cell Transplant Division, Maternal and Child Health Department, Padova University and University Hospital, Padova; [3]Pediatric Hematology Oncology, Department of Mother and Child, Azienda Ospedaliera Universitaria Integrata, Verona; [4]Paediatric Hematology and Oncology Unit, Ospedale Pediatrico Microcitemico, Cagliari; [5]Department of Hematology-Oncology, Anna Meyer Children's Hospital Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Firenze and [6]Clinical Genetics Unit, Department of Women's and Children's Health, University of Padova, Padova, Italy

*GM and FL contributed equally as first authors.

Correspondence:
L. MUSSOLIN - lara.mussolin@unipd.it

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Disclosures

No conflicts of interest to disclose.

Contributions

LM conceived and designed the study. FL and LM supervised data collection and analyses. FL and GM analyzed data and prepared the figures. GM and DR performed laboratory assays. EC and MP were in charge of data pooling, data checking, and statistical analysis. CB, FB and LS performed MLPA analyses. FL, GM and LM wrote the manuscript. SC, RMM, AT and AB provided patient clinical care and revised the manuscript. All authors read and approved the final version of the manuscript.

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Data-sharing statement

All data supporting the findings of this study are included in the main text or in the Online Supplementary Appendix.

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