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

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Disclosures

No conflicts of interests to disclose.

Contributions

L.M. conceived and designed the study; F.L. and L.M. supervised data collection and analyses; F.L. and G.M. analyzed data and prepared figures; G.M. and D.R. performed laboratory assays; E.C. and M.P. were in charge of data pooling, data checking and statistical analysis; C.B., F.B. and L.S. performed MLPA analyses; F.L., G.M. and L.M. wrote the manuscript. S.C., R.M.M., A.T. and A.B. provided patient clinical care and revised the manuscript. All authors read and approved the final version of the manuscript.

Data-sharing statement

All data that supports the findings of this study are included in the main text or in the supplementary material of this article.

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Despite current chemotherapy regimens are extremely effective in curing Burkitt lymphoma (BL) children, the outcome for patients with primary refractory or relapsed disease still remains very poor, with fewer than 30% of them 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 anti CD20 addition 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 and, 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 identifying patients with increased risk of relapse/resistance, 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, two large independent studies demonstrated, by univariate analysis, that *TP53* abnormalities define clinical risk groups also within 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 ritux 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. EBV detection and copy number evaluation was assessed by performing

Real Time PCR on DNA samples from all cases. DNA from EBV-positive Namalwa BL cell line was used to produce a calibration curve with known EBV copy numbers (Namalwa harbors two integrated EBV copies/cell)[10]. Among our cohort, only 9% of patients was EBV-positive.

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 sequence 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 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 five had copy number variations (CNV). As for the 15 cases with biallelic abnormalities, four 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 discriminating between monoallelic/biallelic genomic lesions.

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

cohort were mostly represented by missense mutations[11]. Indeed, of the 102 identified mutations, 93 were missense, five were nonsense, two were in-frame deletions, one was a frameshift mutation and one involved a splicing donor site (Figure 1). Moreover, 13/87 patients showed two coexisting mutations, 1/87 showed three and 17/87 showed only the mutated allele (Supplementary Table 1). 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 three cases, R273H in five cases).

Overall, the presence of *TP53* mutations was associated with a significantly inferior outcome (Figure 2A, Supplementary Figure 1A). 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, Supplementary Figure 1B).

As for the most frequently detected variants, the R175H mutation beard the same prognostic impact of other mutations affecting exon 5 (Figure 1C, Supplementary Figure 1C), whereas the R248Q substitution was significantly associated with a better outcome: patients bearing the R248Q substitution showed a PFS% and OS% very similar to patients with *wild-type TP53* as compared to patients bearing the R248W or other mutations affecting different residues in exon 7 (Figure 1D, Supplementary Figure 1D). Only 3/19 EBV positive cases showed TP53 mutation; all EBV positive patients obtained a continuous clinical remission.

Noteworthy, 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 patients risk-based stratification and that rituximab is effective in improving the cure rates of this aggressive pediatric lymphoma[8].

MDD ad 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)(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 were performed by Ig rearrangements[14].

This analysis did not show prognostic significance. This seems in contrast with 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 injections,

MDD-positive patients showed a significantly inferior outcome compare to MDD-negative patients (Supplementary Figure 2A-B), whereas patients who received rituximab displayed similar PFS% both in the presence or absence of MDD (Supplementary Figure 2C-D), suggesting that rituximab addition to chemotherapy might overcome the prognostic impact of molecular disease dissemination.

However, in order to understand if minimal disease dissemination might improve risk stratification in *TP53* mutated patients, we analyzed the combination of these two parameters and we identified a group of BL patients at very high risk of treatment failure (p-value 0.01, Figure 2E; Supplementary Figure 2E). Indeed, patients both MDD-positive 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-value 0.02, Figure 2F; Supplementary Figure 2F). Among them, all patients performed uniform 6 chemotherapy courses except one who added anti-CD20. Three out six 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 consider 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 for treatment decisions.

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

Patient characteristics		# Patients	# Events	3-y PFS % (SE%)	Univariate *p*-Value	Multivariate *p*-Value	Hazard Ratio (95% CI)
Gender	Male	180	24	86 (3)	0.088	n.s.	
	Female	34	8	76 (7)			
Median age (years)	<7,7	111	14	87 (3)	0.293	n.s.	
	≥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	n.s.	
	4 and C	166	30	81 (3)			
Stage°	1-2	38	1	97 (3)	0.0259	n.s.	
	3-4	176	31	82 (3)			
MDD	Neg	122	14	88 (3)	0.109	n.s.	
	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; 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).

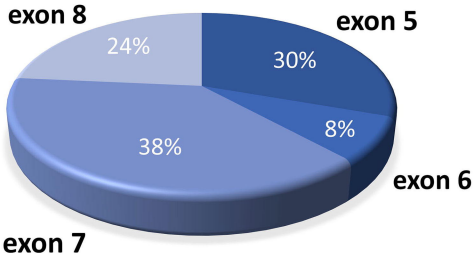
*BM involvement was defined based on smear morphological examination; °St Jude staging system[15]; [#]Risk group 1-4 was defined according to the treatment protocols of the patients i.e. AIEOP LNH97[5] and risk group B-C according to Inter B-NHL Ritux 2010[4].

Figure legends

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 BL patients and their classification.

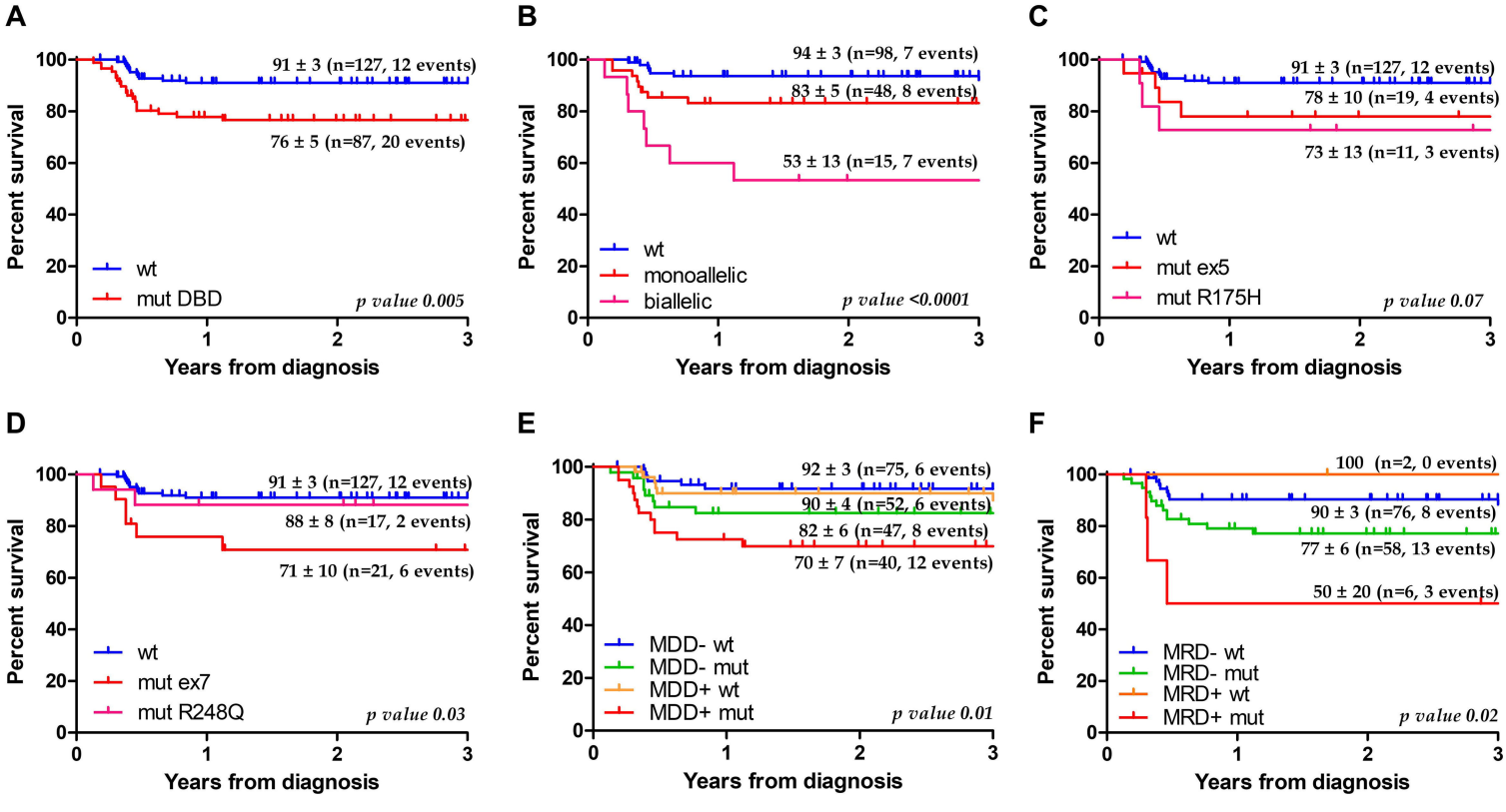
Figure 2. **Progression free survival (PFS) according to TP53 mutational status, alone and in combination with disease dissemination.** 3-year PFS% according to *TP53* DNA binding domain (DBD) mutational status (A), the presence of biallelic/monoallelic mutations on DBD (B) or the presence of specific hot-spot mutations in exons 5 and 7 (C-D). Panels E and F show the combined significance of *TP53* mutations and MDD or MRD, respectively.

Figure 1


exon 8
24%
exon 5
30%
8%
38%
exon 6
exon 7

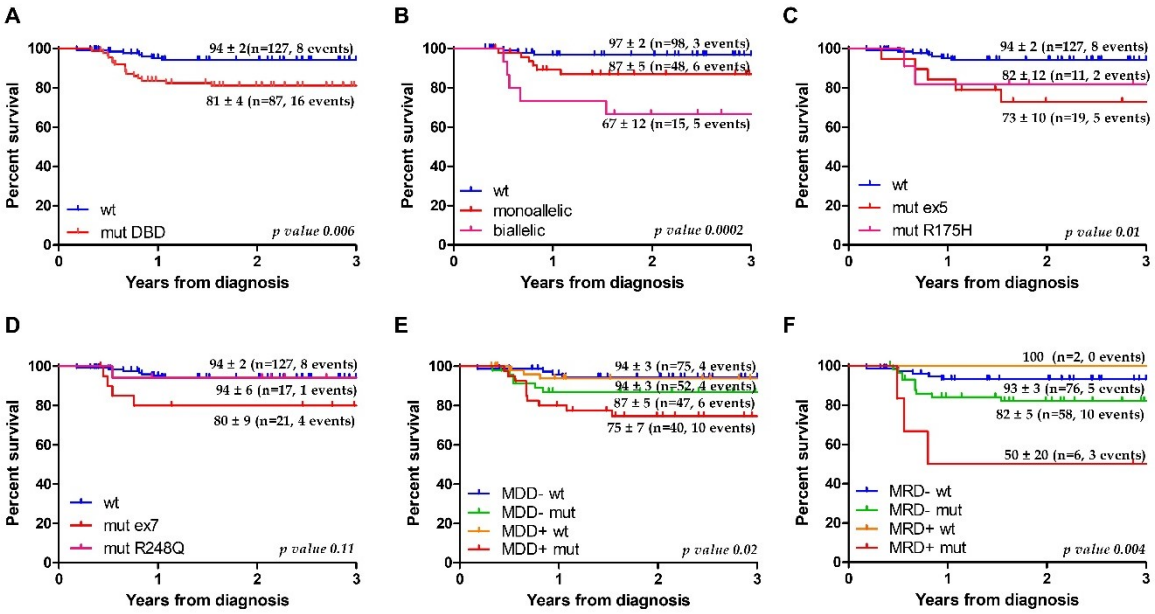
missense mutations
nonsense mutations
in-frame deletion
frameshift
SNV
p.R248W/Q
p.R175H
p.R273C/H
p.S127F
p.M133L/K
p.C135R/F
p.C141Y
p.V143M
p.W146*
p.D148E
p.P151S
p.P152L
p.A161S
p.Y163H
p.V173M
p.C176F
p.P177S
p.H179R
p.R181C
p.N200Tfs*38
p.T211A
p.R213*
p.R213L
p.V218A
p.V218_P219L
p.Y220H
splicing donor
p.I232T
p.Y234D/N
p.Y236N/H
p.M237K
p.C238*
p.N239D
p.G245D/S
p.N247I
p.T253_I255del
p.L257P
p.G266V
p.V272E
p.V274A/F
p.C275Y
p.P278S
p.D281/NH
p.R282W
p.E286K
p.R306*
N-
TAD I
TAD II
PRD
DBD
HD
OD
-C
1
40
67
98
293
326
393

Figure 2

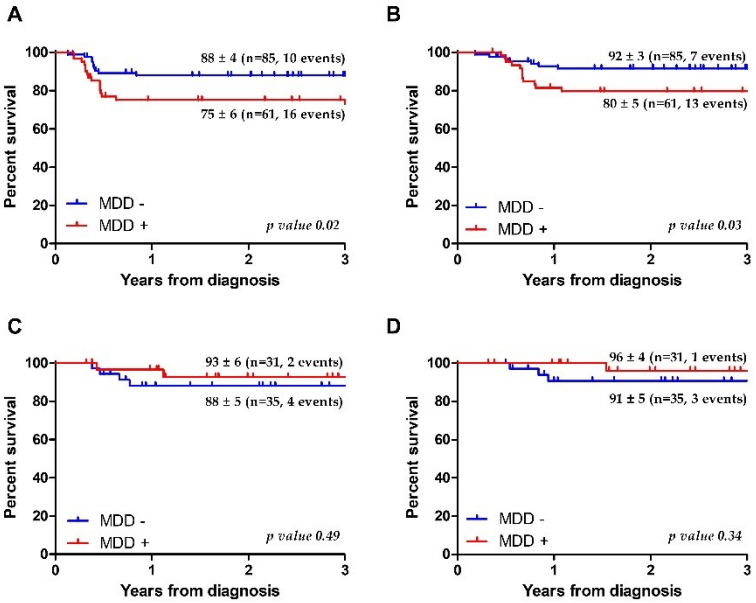

A
Percent survival
Years from diagnosis
91 ± 3 (n=127, 12 events)
76 ± 5 (n=87, 20 events)
wt
mut DBD
p value 0.005
B
Percent survival
Years from diagnosis
94 ± 3 (n=98, 7 events)
83 ± 5 (n=48, 8 events)
53 ± 13 (n=15, 7 events)
wt
monoallelic
biallelic
p value <0.0001
C
Percent survival
Years from diagnosis
91 ± 3 (n=127, 12 events)
78 ± 10 (n=19, 4 events)
73 ± 13 (n=11, 3 events)
wt
mut ex5
mut R175H
p value 0.07
D
Percent survival
Years from diagnosis
91 ± 3 (n=127, 12 events)
88 ± 8 (n=17, 2 events)
71 ± 10 (n=21, 6 events)
wt
mut ex7
mut R248Q
p value 0.03
E
Percent survival
Years from diagnosis
92 ± 3 (n=75, 6 events)
90 ± 4 (n=52, 6 events)
82 ± 6 (n=47, 8 events)
70 ± 7 (n=40, 12 events)
MDD- wt
MDD- mut
MDD+ wt
MDD+ mut
p value 0.01
F
Percent survival
Years from diagnosis
100 (n=2, 0 events)
90 ± 3 (n=76, 8 events)
77 ± 6 (n=58, 13 events)
50 ± 20 (n=6, 3 events)
MRD- wt
MRD- mut
MRD+ wt
MRD+ mut
p value 0.02

Supplementary material

Supplementary Figure 1. Overall survival probability according to TP53 mutational status, alone and in combination with MDD/MRD. 3-year OS% according to *TP53* DNA binding domain (DBD) mutational status (A), the presence of biallelic/monoallelic mutations on DBD (B) or the presence of specific hot-spot mutations in exons 5 and 7 (C-D). Panels E and F show the combined significance of *TP53* mutations and MDD or MRD, respectively.



Supplementary Figure 2. **Prognostic significance of MDD in BL patients treated with/without rituximab addition to standard chemotherapy.** 3-year PFS% (A) and OS% (B) in BL patients treated with standard chemotherapy without rituximab addition; 3-year PFS% (C) and OS% (D) in BL patients who received rituximab in addition to chemotherapy.



Supplementary Table 1.

Patient	exon 5		exon 6		exon 7		exon 8	
	DNA	protein	DNA	protein	DNA	protein	DNA	protein
BL290					c.695T>C - c.742C>T	p.I232T - p.R248W		
BL292							c.818G>A	p.R273H
BL296	c.524G>A	p.R175H						
BL297*	c.444T>G	p.D148E						
BL300					c.743G>A	p.R248Q		
BL301			c.631A>G	p.T211A				
BL303					c.742C>T	p.R248W		
BL305					c.742C>T	p.R248W		
BL306							c.820G>T	p.V274F
BL307					c.712T>A	p.C238*		
BL309							c.844C>T	p.R282W
BL310	c.524G>A	p.R175H					c.841G>C	p.D281H
BL311*	c.524G>A	p.R175H						
BL312					c.743G>A	p.R248Q		
BL314	c.524G>A	p.R175H					c.832C>T	p.P278S
BL315							c.818G>A	p.R273H
BL319			c.673T>G	splicing donor				
BL321*	c.437G>A	p.W146*						
BL323	c.524G>A	p.R175H						
BL324					c.706T>C	p.Y236H		
BL325	c.524G>A	p.R175H						
BL326							c.817C>T	p.R273C
BL327					c.743G>A	p.R248Q		
BL329					c.743G>A	p.R248Q		
BL333			c.653T>C - c.658T>C	p.V218A - p.Y220H	c.743G>A	p.R248Q		
BL337*	c.524G>A	p.R175H						
BL343	c.422G>A	p.C141Y			c.757-765del	p.T253-I255del		
BL344					c.706T>A	p.Y236N		
BL347*					c.743G>A	p.R248Q		
BL348*	c.524G>A	p.R175H						
BL354					c.710T>A	p.M237K		
BL355							c.818G>A	p.R273H
BL356					c.742C>T	p.R248W	c.821T>C	p.V274A
BL358			c.638G>T	p.R213L				
BL359					c.743G>A	p.R248Q		
BL360							c.856G>A	p.E286K
BL362							c.817C>T	p.R273C
BL364	c.404G>T - c.536A>G	p.C135F - p.H179R						
BL365	c.403T>C	p.C135R			c.734G>A	p.G245D		
BL368					c.743G>A	p.R248Q		
BL370*					c.743G>A	p.R248Q		

BL371							c.856G>A	p.E286K
BL376*	c.427G>A	p.V143M						
BL378					c.742C>T	p.R248W		
BL381	c.524G>A	p.R175H					c.916C>T	p.R306*
BL383	c.404G>T	p.C135F						
BL385*	c.380C>T	p.S127F						
BL388					c.743G>A	p.R248Q		
BL390			c. 652GTGCC>CT	p.V218_P219L				
BL391*	c.451C>T	p.P151S						
BL393	c.527G>T	p.C176F						
BL394*							c.844C>T	p.R282W
BL396	c.487T>C	p.Y163H			c.715A>G	p.N239D		
BL402					c.742C>T	p.R248W		
BL407			c.598-622del	p.N200Tfs*38				
BL413					c.743G>A	p.R248Q		
BL415*					c.743G>A	p.R248Q		
BL418*	c.517G>A	p.V173M						
BL420							c.821T>C	p.V274A
BL422					c.700T>C	p.Y234H		
BL426	c.397A>C	p.M133L					c.916C>T	p.R306*
BL428*					c.743G>A	p.R248Q		
BL430*					c.770T>C	p.L257P		
BL432	c.524G>A	p.R175H					c.818G>A	p.R273H
BL434							c.797G>T	p.G266V
BL439							c.818G>A	p.R273H
BL442							c.824G>A	p.C275Y
BL443							c.817C>T	p.R273C
BL447	c.455C>T	p.P152L					c.841G>A	p.D281N
BL448					c.742C>T	p.R248W		
BL452					c.743G>A	p.R248Q		
BL454	c.541C>T	p.R181C						
BL457					c.710T>A	p.M237K		
BL463			C.637C>T	p.R213*				
BL464	c.481G>T	p.A161S						
BL472	c.529C>T	p.P177S			c.743G>A	p.R248Q		
BL475					c.700T>G	p.Y234D		
BL477*	c.524G>A	p.R175H						
BL478	c.398T>A	p.M133K						
BL482							c.844C>T	p.R282W
BL485					c.740A>T	p.N247I		
BL486	c.527G>T	p.C176F						
BL488*					c.743G>A	p.R248Q		
BL493					c.743G>A	p.R248Q		
BL500					c.742C>T	p.R248W		

BL502							c.815T>A	p.V272E
BL504					c.733G>A	p.G245S		

*** homozygous mutation**