BCL2 mutations do not confer adverse prognosis in follicular lymphoma patients treated with rituximab

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Authors:

Sarah Huet [1,2*], Edith Szafer-Glusman[3*], Bruno Tesson[4], Luc Xerri[5], Wayne J. Fairbrother[3], Kiran Mukhyala[3], Chris Bolen[3], Elizabeth Punnoose[3], Laurie Tonon[6], Catherine Chassagne-Clément[7], Pierre Feugier[8], Alain Viari[6,9], Fabrice Jardin[10], Gilles Salles [2], Pierre Sujobert [1,2]

* Equal contributors

Affiliations:

[1]Laboratoire d'hématologie, Hospices Civils de Lyon, 69495 Pierre Bénite cedex, France.
[2]INSERM1052, CNRS 5286, Université Claude Bernard, Faculté de Médecine Lyon-Sud Charles Mérieux, Université de Lyon, 69495 Pierre Bénite cedex, France.
Service d'hématologie clinique, Hospices Civils de Lyon, 69495 Pierre Bénite cedex, France.
[3]Genentech, South San Francisco, California.
[4]Institut Carnot CALYM, Pierre Bénite, France
[5]Aix-Marseille University and Institut Paoli-Calmettes, Marseille
[6]Synergie Lyon Cancer, Plateforme de bioinformatique 'Gilles Thomas', Centre Léon Bérard, Lyon, France.
[7]Department of Pathology, Centre Léon Bérard, Lyon, France.
[8]Nancy University Hospital, Department of Hematology, Nancy, France.
[9]Equipe Erable, INRIA Grenoble-Rhône-Alpes, Montbonnot-Saint Martin, France.
[10]Department of Hematology, Henri Becquerel Comprehensive Cancer Center and Normandie Univ, UNIROUEN, Inserm U1245, Team "Genomics and biomarkers in lymphoma and solid tumors", Rouen, France

Corresponding author:

Pierre Sujobert

Service d'hématologie biologique. Centre hospitalier Lyon Sud.

165 chemin du grand Revoyet. 69310 Pierre Bénite, France

pierre.sujobert@chu-lyon.fr / Phone number: (0033) 4788615 / Fax: (0033) 478864104

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Abstract

BCL2 mutations have been suggested to confer an adverse prognosis to follicular lymphoma (FL) patients, but their prognostic value has not been assessed in patients treated with a rituximab-containing regimen. Here we evaluated the prognostic value of *BCL2* mutations in a large prospective cohort of 252 FL patients treated with immunochemotherapy in the PRIMA randomized trial. Using a DNA-targeted sequencing approach, we detected amino acid altering mutations in 135 patients (54%) and showed that these mutations were probably mediated by the over-activation of AICDA (Activation-induced cytidine deaminase) in the context of the t(14;18) translocation. The BCL2 variants identified in PRIMA patients affected the BH1, BH2 and BH3 functional motifs at a lower frequency than the N-terminus and flexible loop domain (FLD), with mostly conservative aminoacid changes. With a median follow-up of 6.7 years, we did not observe any impact of *BCL2* mutations either on overall survival or progression-free survival.

Introduction

The clinical course of follicular lymphoma (FL) is heterogeneous, and the best clinical predictor of outcome is the FLIPI score[1,2]. It is increasingly recognised that genomic alterations can influence patients' outcome, which led recently to the integration of genes mutational status in the prognostic stratification of FL patients[3].

The t(14;18) translocation is the hallmark of FL, resulting in overexpression of the *BCL2* oncogene. *BCL2* is also frequently mutated in FL[4], and *BCL2* mutations were recently shown to be associated with an increased risk of transformation and risk of death due to lymphoma[5]. Mutation analysis in the Correia's retrospective study used Sanger sequencing in a patient cohort mostly treated in the absence of rituximab. Here we use next-generation sequencing to identify *BCL2* mutations and assess their prognostic value in a large prospective cohort of 252 FL patients treated in the PRIMA Phase III trial that evaluated Rituximab maintenance after immunochemotherapy as first line treatment in FL[6].

Methods

This study was conducted in accordance with the Declaration of Helsinki. All patients signed a consent form for participation in specific biological studies.

Tumour biopsy specimens were obtained at FL diagnosis from 252 high tumour burden patients included in the PRIMA trial[6]. DNA was extracted from FFPE tissues (n=98) or fresh-frozen tissues (FF, n=154). A DNA-targeted sequencing approach using the FoundationOne Heme platform was performed as described previously[7] (mean coverage 797x). Lymphoma-specific variants identified in the *BCL2* gene were filtered to remove known polymorphisms (dbSNP database v137) and possible sequencing artifacts by exclusion of variants with variant allele frequency (VAF) under 5% (n=18). These data also provided information on *IGH-BCL2* rearrangement.

RNA-seq data available for 143 of the 252 patients was used to assess expression levels of *AICDA* transcript.

The correlation between *BCL2* mutation status and clinical or biological characteristics were assessed using Fisher's exact test. Differential expression levels or VAF were compared using the Mann-Whitney test. Progression-free survival (PFS) and overall survival (OS), defined by international criteria[8], were estimated by the Kaplan-Meier product limit method and compared by log-rank test.

Results and Discussion

One hundred and thirty five patients (54%) harboured at least one amino acid altering mutation, consisting in single nucleotide variations (SNVs) (n=294) or small in-frame insertions (n=3) (supplemental table 1). This proportion was slightly higher in fresh frozen tissue (FF) (89/154, 58%) than in FFPE samples (46/98, 47%), possibly reflecting a lower sensitivity of sequencing to detect mutations on DNA extracted from FFPE tissues. Nevertheless, the frequency of *BCL2* coding mutations at FL diagnosis is higher than the previously reported 12% with Sanger sequencing[5], and similar to that reported with Next-Generation Sequencing techniques[3,9]. As described in DLBCL [5,10], multiple (≥2) non-synonymous mutations in *BCL2* were identified in 71 patients (range: 0-17 mutations per sample). We evaluated the accuracy of our analytic pipeline by comparing the findings of our targeted approach with the findings of whole exome sequencing of tumoral and normal tissues for a subgroup of 10 patients. Overall, the false discovery rate and false negative rate for FoundationOne Heme DNA targeted sequencing were low: one variant called by FMI was also present in germline DNA (1/28 false positive, 3,6%), and one mutation at a splice-site was not detected by FMI (1/28 false negative, 3,6%).

Non-synonymous SNVs were located throughout the second exon, predominantly in the flexible-loop regulatory domain, whereas the third exon of the longest (alpha) isoform encoding the transmembrane domain was spared, consistent with previous observations[5] (Figure 1A). Although mutations were frequent in the *BCL2* gene, we did not detect frame-shifts and stop-codons in the alpha isoform, which could result in loss of the functional protein. Two mutations occurred at the terminal stop-codon of the beta isoform that lacks the C-terminus transmembrane domain; the functional effect of these mutations is unclear. Mutations in the functional BH1, BH2 and BH3 motifs were rare in our cohort occurring at an average frequency of 0.5 variants per amino acid, compared to 2-4.7 variants per amino acids in the FLD and N-terminus domains (Supplemental Table 2). Mutations in the BH3-binding groove of BCL-2 were largely conservative in nature and not expected to affect binding to pro-apoptotic proteins based on structural analysis of the BCL-2 protein bound to the BAX BH3 peptide (Supplemental Figure 1). This is in agreement with computational analysis suggesting that none of the mutations observed in BCL-2 are predicted to have deleterious effects (MutationAssessor[11,12])

(Supplemental Table 3). However, further *in vitro* functional studies and clinical results from BCL-2 inhibitors' trials will warrant the functional consequences of these mutations.

Variant Allele Frequency (VAF) depends on the sample's tumour content, the hetero- or homozygous status, the copy number variation of the considered locus, and the clonal or subclonal nature of the mutation. The VAF of *BCL2* mutations were highly heterogeneous (range: 0.06-0.59; median: 0.26) in our cohort. By studying the VAF ratio (defined as the ratio of the VAF compared to the higher *BCL2* mutation VAF of the same sample) in 71 patients carrying multiple *BCL2* mutations, we identified 29% of the mutations with a low VAF ratio. Although tumor cell content may also account for this variability, these results indicate that most *BCL2* mutations accumulate in subclones, reflecting ongoing genomic instability in FL (Figure 1B)[13].

An *IGH-BCL2* rearrangement was identified in 92% (141/154) and 42% (41/98) of the FF and FFPE samples, respectively, which led us to limit the following analysis on the subgroup of FF samples. The presence of mutations in the *BCL2* sequence was associated with the presence of *IGH-BCL2* rearrangements (Figure 1C), as previously reported[14,15], suggesting that *BCL2* mutations might be a consequence of the juxtaposition of the *BCL2* gene in the vicinity of the *IGH* locus caused by the t(14;18) translocation. The activation of activation-induced cytidine deaminase (AICDA) might also account for a higher frequency of *BCL2* mutations[5,15]. Indeed, our RNAseq data revealed a slightly higher expression of *AICDA* mRNA in *BCL2* mutated compared to wild-type samples (p=0.003) (Figure 1D). Moreover, 51% of the SNVs were transitions (C to T or G to A) and 24% occurred at preferred AICDA target sequences RGYW/WRCY.

Finally, we found no association between any of the clinical characteristics at diagnosis and *BCL2* mutation status (Supplemental Table 4). With a median follow-up of 6.7 years, we were unable to detect any significant difference in PFS or OS between mutated and wild-type patients (Figure 2A and 2B). The rate of histological transformation was not impacted by the mutational status of *BCL2* (WT: 2/19 (10.5%) *versus BCL2* mutated: 4/29 (13.8%), ns) in patients who had a biopsy taken at progression. This remained true when analyzing survival in rituximab maintenance *versus* observation arms separately (Supplemental Figure 2). The results contrast with previous findings[5]; the lack of survival effect was maintained even when only mutations common to both studies were analyzed (not shown). Patients from both studies were treated

with chemotherapy, where *BCL2* mutations may contribute to resistance to therapy[16,17]. On the contrary, all PRIMA patients were treated with a rituximab-based regimen, and one possible interpretation is that the addition of rituximab could explain the improvement in outcome of *BCL2* mutated patients. Indeed, rituximab is mainly acting through complement dependent cytotoxicity and antibody dependent cellular cytotoxicity[18], which are not mediated by the cell intrinsic apoptosis pathway, and hence might be independent of BCL2 function. This hypothesis could be tested by the retrospective analysis of the prognostic value of BCL2 mutation in randomized clinical trials of rituximab vs placebo in FL. Of note, there are a number of other differences between the two studies, which preclude the identification of a single factor to explain the conflicting results. These include the high rate of transformation in the patient population from the Correia study in comparison to PRIMA (21% in PRIMA[19] *versus* 44% in Correia's study), differences in mutation prevalence, as a result of the different mutation detection methodologies (54% in PRIMA *versus* 12% in Correia's), and analysis endpoints (PFS and OS in PRIMA *versus* risk of transformation and death from lymphoma in Correia's).

Altogether, our data show that *BCL2* mutations are frequent at FL diagnosis, probably mediated by the overactivation of AICDA in the context of the t(14;18) translocation. *BCL2* mutations in FL patients treated with current standard-of-care rituximab failed to demonstrate an association with survival. The t(14;18) translocation is also the key mechanism leading to the characteristic overexpression of BCL-2 protein in FL; the prognostic impact of BCL-2 expression in FL patients from PRIMA will be further investigated.

Acknowledgements

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Authorship Contributions

SH, ESG, EP, GS and PS designed the study and wrote the manuscript. CB, LT, BT and AV performed the bioinformatics analysis, WJF and KM performed the structural analysis, LX, CCC, PF, FJ and GS contributed clinical and pathological data.

All authors contributed to reviewing the manuscript.

Disclosure of Conflicts of Interest

ES, WJF, KM, CB, and EP are employees of Genentech, Inc.

References

1. Solal-Céligny P, Roy P, Colombat P, et al. Follicular lymphoma international prognostic index. *Blood*. 2004;104(5):1258-1265. doi:10.1182/blood-2003-12-4434.

2. Federico M, Bellei M, Marcheselli L, et al. Follicular lymphoma international prognostic index 2: a new prognostic index for follicular lymphoma developed by the international follicular lymphoma prognostic factor project. *J Clin Oncol Off J Am Soc Clin Oncol*. 2009;27(27):4555-4562. doi:10.1200/JCO.2008.21.3991.

3. Pastore A, Jurinovic V, Kridel R, et al. Integration of gene mutations in risk prognostication for patients receiving first-line immunochemotherapy for follicular lymphoma: a retrospective analysis of a prospective clinical trial and validation in a population-based registry. *Lancet Oncol*. 2015;16(9):1111-1122. doi:10.1016/S1470-2045(15)00169-2.

4. Pasqualucci L, Khiabanian H, Fangazio M, et al. Genetics of follicular lymphoma transformation. *Cell Rep*. 2014;6(1):130-140. doi:10.1016/j.celrep.2013.12.027.

5. Correia C, Schneider PA, Dai H, et al. BCL2 mutations are associated with increased risk of transformation and shortened survival in follicular lymphoma. *Blood*. 2015;125(4):658-667. doi:10.1182/blood-2014-04-571786.

6. Salles G, Seymour JF, Offner F, et al. Rituximab maintenance for 2 years in patients with high tumour burden follicular lymphoma responding to rituximab plus chemotherapy (PRIMA): a phase 3, randomised controlled trial. *Lancet*. 2011;377(9759):42-51. doi:10.1016/S0140-6736(10)62175-7.

7. He J, Abdel-Wahab O, Nahas MK, et al. Integrated genomic DNA/RNA profiling of hematologic malignancies in the clinical setting. *Blood*. January 2016:blood-2015-08-664649. doi:10.1182/blood-2015-08-664649.

8. Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. *J Clin Oncol Off J Am Soc Clin Oncol*. 1999;17(4):1244.

9. Okosun J, Bödör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. *Nat Genet*. 2014;46(2):176-181. doi:10.1038/ng.2856.

10. Schuetz JM, Johnson NA, Morin RD, et al. BCL2 mutations in diffuse large B-cell lymphoma. *Leukemia*. 2012;26(6):1383-1390. doi:10.1038/leu.2011.378.

11. Gnad F, Baucom A, Mukhyala K, Manning G, Zhang Z. Assessment of computational methods for predicting the effects of missense mutations in human cancers. *BMC Genomics*. 2013;14 Suppl 3:S7. doi:10.1186/1471-2164-14-S3-S7.

12. Reva B, Antipin Y, Sander C. Predicting the functional impact of protein mutations: application to cancer genomics. *Nucleic Acids Res*. 2011;39(17):e118-e118. doi:10.1093/nar/gkr407.

13. Green MR, Gentles AJ, Nair RV, et al. Hierarchy in somatic mutations arising during genomic evolution and progression of follicular lymphoma. *Blood*. 2013;121(9):1604-1611. doi:10.1182/blood-2012-09-457283.

14. Burkhard R, Bhagat G, Cogliatti SB, et al. BCL2 mutation spectrum in B-cell non-Hodgkin lymphomas and patterns associated with evolution of follicular lymphoma. *Hematol Oncol*. February 2014. doi:10.1002/hon.2132.

15. Lohr JG, Stojanov P, Lawrence MS, et al. Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc Natl Acad Sci U S A*. 2012;109(10):3879-3884. doi:10.1073/pnas.1121343109.

16. Takahashi M, Saito H, Atsukawa K, Ebinuma H, Okuyama T, Ishii H. Bcl-2 prevents doxorubicin-induced apoptosis of human liver cancer cells. *Hepatol Res Off J Jpn Soc Hepatol*. 2003;25(2):192-201.

17. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood*. 1993;81(1):151-157.

18. Pérez-Callejo D, González-Rincón J, Sánchez A, Provencio M, Sánchez-Beato M. Action and resistance of monoclonal CD20 antibodies therapy in B-cell Non-Hodgkin Lymphomas. *Cancer Treat Rev*. 2015;41(8):680-689. doi:10.1016/j.ctrv.2015.05.007.

19. Sarkozy C, Trneny M, Xerri L, et al. Risk Factors and Outcomes for Patients With Follicular Lymphoma Who Had Histologic Transformation After Response to First-Line Immunochemotherapy in the PRIMA Trial. *J Clin Oncol*. June 2016:JCO657163. doi:10.1200/JCO.2015.65.7163.

Figure Legends

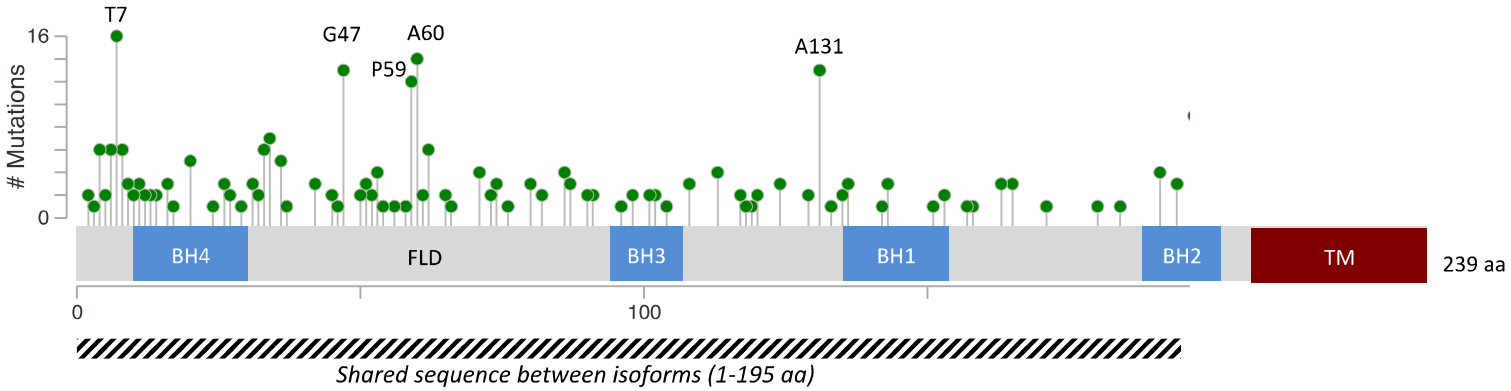
Figure 1: Description of BCL2 mutations in follicular lymphoma A: Schematic diagram of the BCL2 protein (isoform alpha NM_000657.2, upper part and isoform beta NM_000633.2, lower part) showing BCL2 homology domains and distribution of amino acid alterations. B: Distribution of clonal and subclonal mutations in patients with multiple *BCL2* mutations. The VAF fraction is the ratio of the VAF of secondary mutations in *BCL2* divided by the higher VAF of *BCL2* mutation in the same sample. A VAF ratio under 75% is considered as subclonal. C: Proportion of mutated (including both synonymous and non-synonymous SNVs and small insertions) and wild-type cases with *IGH-BCL2* rearrangement among the FFT samples. The presence of an *IGH-BCL2* rearrangement was detected in 23% and 81% of the wild type and mutated cases, respectively ($p<0.0001$). D: AICDA transcript level from 143 samples with available expression data (RNA-seq), according to their BCL2 mutational status. BH, BCL2 Homology domain; FLD, Flexible Loop regulatory Domain; TM, transmembrane domain.

Figure 2: Outcome of patients with *BCL2* non-synonymous mutation. Progression-free survival (A) and overall survival (B) in the entire cohort since the registration date in PRIMA trial. No differences in PFS and OS were detected between mutated (ie non-synonymous coding mutations) and non-mutated (ie without non-synonymous coding mutations) patients by log-rank test.

Figure 1

A.

BCL2 isoform alpha:



BCL2 isoform beta:

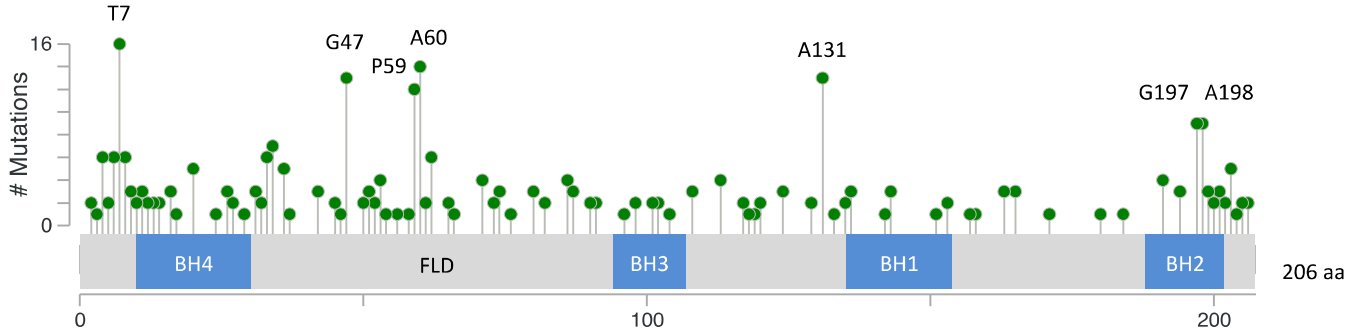
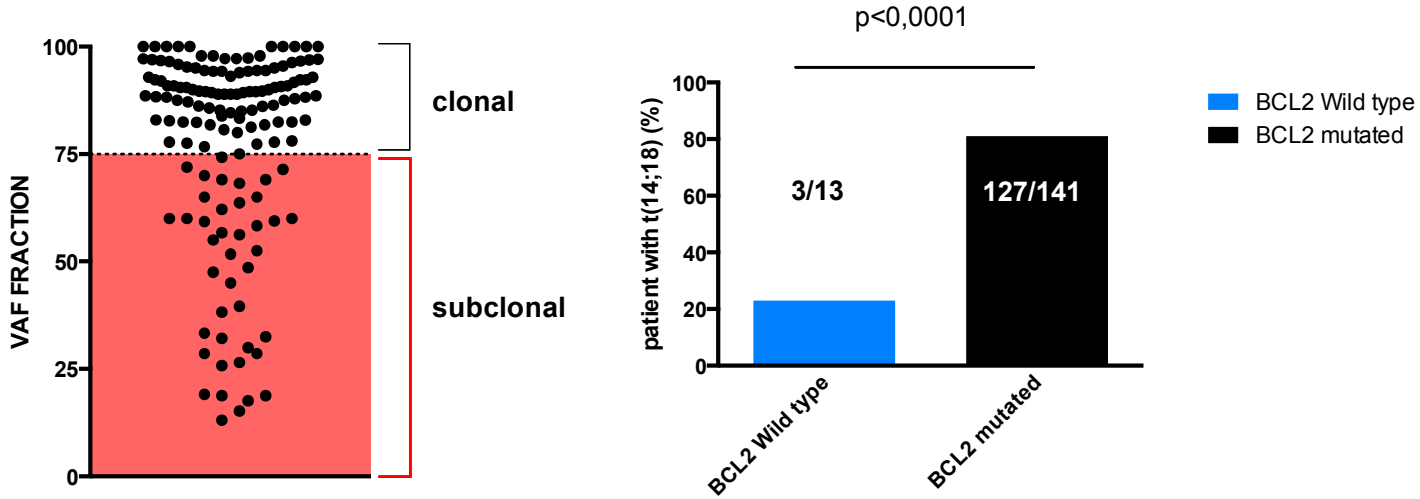


Figure 1 (continued)

B.



D.

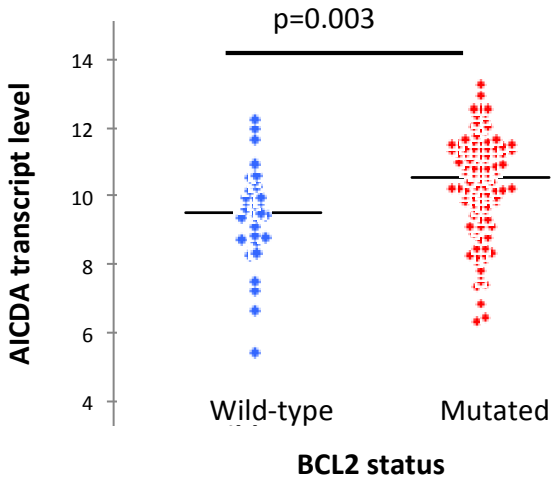
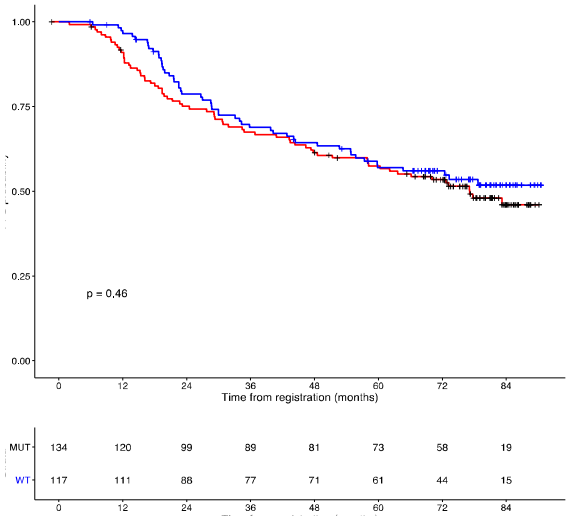
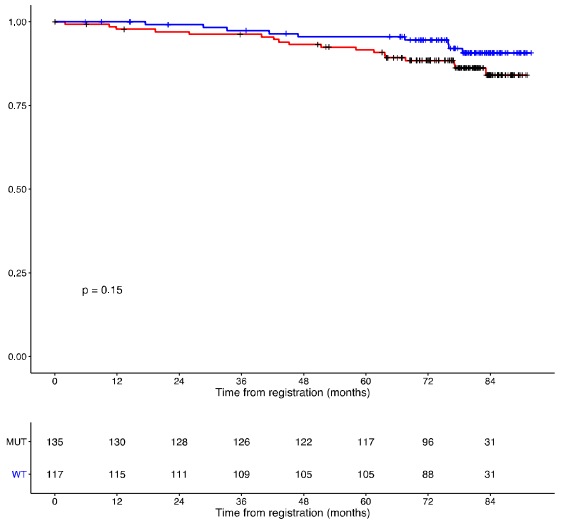


Figure 2



Time from registration to progression or death (PFS, months)



Time from registration to death (OS, months)

BCL2 mutated
BCL2 wild-type

Supplementary table 1

Sample ID	PROTEIN_CHANGE	CDS_CHANGE	GENOME_POSITION	COVERAGE
600887	A2T	4G>A	chr18:60985896	1088
600219	A2T	4G>A	chr18:60985896	1040
600696	H3Y	7C>T	chr18:60985893	578
600398	A4T	10G>A	chr18:60985890	679
700603	A4G	11C>G	chr18:60985889	737
600448	A4V	11C>T	chr18:60985889	633
600682	A4V	11C>T	chr18:60985889	1291
700611	A4G	11C>G	chr18:60985889	791
700657	A4G	11C>G	chr18:60985889	605
600243	G5E	14G>A	chr18:60985886	534
700629	G5E	14G>A	chr18:60985886	1179
600266	R6K	17G>A	chr18:60985883	434
600222	R6T	17G>C	chr18:60985883	882
600398	R6T	17G>C	chr18:60985883	692
600765	R6T	17G>C	chr18:60985883	754
600886	R6T	17G>C	chr18:60985883	1444
601088	R6T	17G>C	chr18:60985883	1044
600837	T7A	19A>G	chr18:60985881	765
700621	T7P	19A>C	chr18:60985881	680
700652	T7A	19A>G	chr18:60985881	756
600524	T7M	20C>T	chr18:60985880	269
600012	T7I	20C>T	chr18:60985880	812
600222	T7M	20C>T	chr18:60985880	886
600375	T7I	20C>T	chr18:60985880	714
600715	T7R	20C>G	chr18:60985880	666
600739	T7I	20C>T	chr18:60985880	908
700548	T7M	20C>T	chr18:60985880	670
700584	T7M	20C>T	chr18:60985880	710
600524	T7M	21A>G	chr18:60985879	269
600222	T7M	21A>G	chr18:60985879	886
600715	T7R	21A>G	chr18:60985879	666
700548	T7M	21A>G	chr18:60985879	670
700584	T7M	21A>G	chr18:60985879	710
600182	G8W	22G>T	chr18:60985878	764
600796	G8R	22G>A	chr18:60985878	1494
600184	G8A	23G>C	chr18:60985877	830
700620	G8K	22G>A	chr18:60985877	905
700620	G8K	23G>A	chr18:60985876	905
700611	Y9N	25T>A	chr18:60985875	839
700620	G8K	24G>A	chr18:60985875	905
700570	Y9_D10insD	25_26insACG	chr18:60985874	755
600102	Y9F	26A>T	chr18:60985874	1293
600414	D10E	30T>G	chr18:60985870	654

601096	D10E	30T>A	chr18:60985870	760
600501	N11Y	31A>T	chr18:60985869	784
601362	N11Y	31A>T	chr18:60985869	778
600737	N11T	32A>C	chr18:60985868	657
600265	R12W	34C>T	chr18:60985866	974
600524	R12W	34C>T	chr18:60985866	268
600746	E13D	39G>C	chr18:60985861	591
600770	E13D	39G>C	chr18:60985861	1027
600496	I14L	40A>C	chr18:60985860	844
600683	I14L	40A>C	chr18:60985860	796
600798	M16V	46A>G	chr18:60985854	795
600865	M16L	46A>C	chr18:60985854	696
700338	M16V	46A>G	chr18:60985854	655
700611	K17R	50A>G	chr18:60985850	824
700679	H20R	59A>G	chr18:60985841	688
600402B	H20R	59A>G	chr18:60985841	755
600414	H20Q	60T>A	chr18:60985840	614
600726	H20Q	60T>G	chr18:60985840	686
600842	H20Q	60T>G	chr18:60985840	616
600737	S24A	70T>G	chr18:60985830	720
600217	R26G	76A>G	chr18:60985824	1034
600482	R26K	77G>A	chr18:60985823	656
600798	R26T	77G>C	chr18:60985823	686
600682	G27A	80G>C	chr18:60985820	1341
600736	G27A	80G>C	chr18:60985820	848
601039	E29A	86A>C	chr18:60985814	691
600184	D31G	92A>G	chr18:60985808	629
700599	D31G	92A>G	chr18:60985808	724
700663	D31E	93T>A	chr18:60985807	785
700599	A32T	94G>A	chr18:60985806	724
700550	A32G	95C>G	chr18:60985805	649
600676	G33R	97G>A	chr18:60985803	622
600684	G33R	97G>A	chr18:60985803	303
600217	G33R	97G>A	chr18:60985803	906
600243	G33R	97G>A	chr18:60985803	564
600865	G33V	98G>T	chr18:60985802	438
700432	G33A	98G>C	chr18:60985802	631
600088	D34N	100G>A	chr18:60985800	619
600243	D34Y	100G>T	chr18:60985800	559
600495	D34N	100G>A	chr18:60985800	845
600496	D34N	100G>A	chr18:60985800	677
700663	D34N	100G>A	chr18:60985800	739
600400	D34E	102T>A	chr18:60985798	740
600842	D34E	102T>A	chr18:60985798	431
600681	G36D	107g>A	chr18:60985793	902
600847	G36D	107g>A	chr18:60985793	660

601039	G36D	107g>A	chr18:60985793	627
700338	G36D	107g>A	chr18:60985793	536
700366	G36D	107g>A	chr18:60985793	401
601362	A37P	109g>C	chr18:60985791	504
600690	A42P	124g>C	chr18:60985776	566
700565	A42T	124g>A	chr18:60985776	481
600748	A42V	125c>T	chr18:60985775	577
600718	A45P	133g>C	chr18:60985767	724
600737	A45T	133g>A	chr18:60985767	632
700345	P46S	136C>T	chr18:60985764	495
600097	G47D	140G>A	chr18:60985760	660
600370	G47V	140G>T	chr18:60985760	627
600484	G47D	140G>A	chr18:60985760	551
600715	G47D	140G>A	chr18:60985760	594
600736	G47D	140G>A	chr18:60985760	765
600770	G47D	140G>A	chr18:60985760	887
600837	G47D	140G>A	chr18:60985760	630
600860	G47D	140G>A	chr18:60985760	2990
600864	G47A	140G>C	chr18:60985760	1386
700363	G47D	140G>A	chr18:60985760	588
700549	G47D	140G>A	chr18:60985760	588
700652	G47D	140G>A	chr18:60985760	566
800329	G47D	140G>A	chr18:60985760	650
700570	S50A	148T>G	chr18:60985752	565
700549	S50P	148T>C	chr18:60985752	618
600482	S51P	151T>C	chr18:60985749	593
800331	S51A	151T>G	chr18:60985749	824
600834	S51C	152C>G	chr18:60985748	952
700599	Q52R	155A>G	chr18:60985745	771
600118	Q52H	156G>T	chr18:60985744	562
600241	P53S	157C>T	chr18:60985743	2277
600769	P53S	157C>T	chr18:60985743	723
600802	P53S	157C>T	chr18:60985743	1142
700018	P53S	157C>T	chr18:60985743	598
600476	G54E	161G>A	chr18:60985739	689
600713	T56A	166A>G	chr18:60985734	571
600243	H58L	173A>T	chr18:60985727	505
600898	P59S	175C>T	chr18:60985725	344
601054	P59S	175C>T	chr18:60985725	459
600104	P59A	175C>G	chr18:60985725	610
600120	P59A	175C>G	chr18:60985725	820
600398	P59A	175C>G	chr18:60985725	703
600769	P59S	175C>T	chr18:60985725	716
600896	P59A	175C>G	chr18:60985725	657
700432	P59A	175C>G	chr18:60985725	732
700544	P59S	175C>T	chr18:60985725	358

600385	P59L	176C>T	chr18:60985724	362
600396	P59L	175C>T	chr18:60985724	808
600396	P59L	176C>T	chr18:60985723	808
700363	A60S	178G>T	chr18:60985722	649
600310	A60V	179C>T	chr18:60985721	726
601054	A60S	178G>A	chr18:60985721	475
700570	A60V	179C>T	chr18:60985721	575
700603	A60V	179C>T	chr18:60985721	580
600088	A60V	179C>T	chr18:60985721	535
600217	A60V	179C>T	chr18:60985721	1191
600223	A60V	179C>T	chr18:60985721	582
600243	A60G	179C>G	chr18:60985721	482
600690	A60D	179C>A	chr18:60985721	604
600787	A60V	179C>T	chr18:60985721	420
700678	A60V	179C>T	chr18:60985721	647
601054	A60S	179C>G	chr18:60985720	475
600396	A60V	179C>T	chr18:60985720	808
600243	A61T	181G>A	chr18:60985719	488
600398	A61T	181G>A	chr18:60985719	722
601126	S62T	184T>A	chr18:60985716	555
601129	S62T	184T>A	chr18:60985716	617
600219	S62F	185C>T	chr18:60985715	976
600736	S62F	185C>T	chr18:60985715	881
600736	S62F	185C>T	chr18:60985715	881
600860	S62F	185C>T	chr18:60985715	3095
600737	P65S	193C>T	chr18:60985707	698
600860	P65S	193C>T	chr18:60985707	2987
600101	V66A	197T>C	chr18:60985703	446
600126	P71S	211C>T	chr18:60985689	638
600225	P71A	211C>G	chr18:60985689	583
601046	P71S	211C>T	chr18:60985689	618
601096	P71S	211C>T	chr18:60985689	633
600862	Q73E	217C>G	chr18:60985683	1198
700603	Q73R	218A>G	chr18:60985682	563
700603	T74A	220A>G	chr18:60985680	562
600793	T74I	221c>T	chr18:60985679	604
700678	T74S	221c>G	chr18:60985679	681
600222	A76T	226g>A	chr18:60985674	686
601054	A80P	238g>C	chr18:60985662	931
601126	A80G	239c>G	chr18:60985661	562
601129	A80G	239c>G	chr18:60985661	572
600729	A82S	244g>T	chr18:60985656	1213
700432	A82V	245c>T	chr18:60985655	708
600396	L86F	256C>T	chr18:60985644	834
600795	L86F	256C>T	chr18:60985644	1344
600876	L86F	256C>T	chr18:60985644	826

600684	L86R	257T>G	chr18:60985643	314
600398	S87N	260G>A	chr18:60985640	756
600349	S87R	261C>A	chr18:60985639	839
600398	S87N	261C>T	chr18:60985639	756
600220	P90T	268C>A	chr18:60985632	721
600243	P90S	268C>T	chr18:60985632	646
600370	P91L	272C>T	chr18:60985628	789
600402B	P91R	272C>G	chr18:60985628	597
700565	T96A	286A>G	chr18:60985614	594
600684	R98C	292C>T	chr18:60985608	270
600769	R98C	292C>T	chr18:60985608	817
600765	G101V	302G>T	chr18:60985598	699
700663	G101_D102insDDFS	302_303insCGACGACTTCTC	chr18:60985597	930
600058	D102A	305A>C	chr18:60985595	1011
600495	D102A	305A>C	chr18:60985595	1169
601085	F104L	310T>C	chr18:60985590	781
600860	Y108H	322T>C	chr18:60985578	4251
600243	Y108S	323A>C	chr18:60985577	699
700565	Y108F	323A>T	chr18:60985577	668
600336	A113V	338C>T	chr18:60985562	720
600398	A113V	338C>T	chr18:60985562	1000
600726	A113V	338C>T	chr18:60985562	890
700345	A113G	338C>G	chr18:60985562	835
700565	S117R	349A>C	chr18:60985551	712
600865	S117T	350G>C	chr18:60985550	791
600696	Q118R	353A>G	chr18:60985547	713
600480	L119V	355C>G	chr18:60985545	1013
600396	H120Y	358C>T	chr18:60985542	1109
600496	H120Y	358C>T	chr18:60985542	899
600222	F124V	370T>G	chr18:60985530	956
600398	F124L	370T>C	chr18:60985530	970
600126	F124C	371T>G	chr18:60985529	857
601091	R129H	386G>A	chr18:60985514	775
700663	R129H	386G>A	chr18:60985514	1038
700548	A131T	391G>A	chr18:60985509	783
600012	A131V	392C>T	chr18:60985508	892
600088	A131V	392C>T	chr18:60985508	833
600243	A131V	392C>T	chr18:60985508	670
600449	A131D	392C>A	chr18:60985508	1460
600450	A131D	392C>A	chr18:60985508	931
600715	A131D	392C>A	chr18:60985508	699
600749	A131V	392C>T	chr18:60985508	794
600801	A131V	392C>T	chr18:60985508	1046
600842	A131D	392C>A	chr18:60985508	736
600865	A131V	392C>T	chr18:60985508	793

700338	A131V	392C>T	chr18:60985508	776
700679	A131D	392C>A	chr18:60985508	765
600243	V133M	397G>A	chr18:60985503	662
600243	E135D	405G>C	chr18:60985495	646
600370	E135D	405G>T	chr18:60985495	933
600302	E136D	408G>C	chr18:60985492	1020
601089	E136D	408G>C	chr18:60985492	996
700645	E136D	408G>C	chr18:60985492	1152
600415	V142M	424G>A	chr18:60985476	619
601124	N143T	428A>C	chr18:60985472	1285
600184	N143T	428A>C	chr18:60985472	864
600736	N143S	428A>G	chr18:60985472	1041
700338	F151L	451T>C	chr18:60985449	825
700570	F153L	457T>C	chr18:60985443	749
601085	F153I	457T>A	chr18:60985443	907
700550	M157L	469A>T	chr18:60985431	936
600842	C158S	473G>C	chr18:60985427	770
601368	N163S	488A>G	chr18:60985412	816
600765	N163K	489C>G	chr18:60985411	823
601114	N163K	489C>G	chr18:60985411	824
600243	E165K	493G>A	chr18:60985407	760
700656	E165G	494A>G	chr18:60985406	877
601124	E165D	495G>C	chr18:60985405	1086
600898	D171E	513C>G	chr18:60985387	411
700432	Y180F	539A>T	chr18:60985361	933
600243	H184Y	550C>T	chr18:60985350	755
600718	D191N	571G>A	chr18:60985329	969
601041	D191E	573T>A	chr18:60985327	752
600495	D191E	573T>G	chr18:60985327	1044
600402B	D191E	573T>A	chr18:60985327	815
700603	G194D	581G>A	chr18:60985319	766
600222	G194D	581G>A	chr18:60985319	925
600865	G194D	581G>A	chr18:60985319	802
600506	G197S	589G>A	chr18:60985311	314
600301	G197S	589G>A	chr18:60985311	854
600472	G197S	589G>A	chr18:60985311	775
600495	G197S	589G>A	chr18:60985311	960
600501	G197S	589G>A	chr18:60985311	795
600696	G197S	589G>A	chr18:60985311	678
601089	G197S	589G>A	chr18:60985311	929
700366	G197S	589G>A	chr18:60985311	574
700432	G197S	589G>A	chr18:60985311	811
600058	A198T	592G>A	chr18:60985308	941
600336	A198T	592G>A	chr18:60985308	625
600796	A198T	592G>A	chr18:60985308	1373
600864	A198S	592G>T	chr18:60985308	1338

700621	A198T	592G>A	chr18:60985308	766
600726	A198E	593C>A	chr18:60985307	742
600865	A198V	593C>T	chr18:60985307	781
700599	A198V	593C>T	chr18:60985307	999
600402B	A198V	593C>T	chr18:60985307	746
600243	L199P	596T>C	chr18:60985304	599
600787	L199P	596T>C	chr18:60985304	578
700363	L199P	596T>C	chr18:60985304	769
601114	G200S	598G>A	chr18:60985302	726
600126	G200D	599G>A	chr18:60985301	739
700432	D201N	601G>A	chr18:60985299	763
700454	D201G	602A>G	chr18:60985298	687
600243	D201E	603T>A	chr18:60985296	557
600737	V202L	604G>T	chr18:60985296	798
600243	V202M	604G>A	chr18:60985295	557
600012	S203N	608G>A	chr18:60985292	744
600243	S203N	608G>A	chr18:60985292	544
600398	S203N	608G>A	chr18:60985292	819
600713	S203N	608G>A	chr18:60985292	787
700432	S203T	608G>C	chr18:60985292	747
700679	L204R	611T>G	chr18:60985289	632
600221	G205A	614G>C	chr18:60985286	504
700432	G205A	614G>C	chr18:60985286	717
700550	*206W	618A>G	chr18:60985282	766
700611	*206fs*1+	618_618delAGGCCACAGGTCCGA	chr18:60985267	747

Supplementary table 2

ALPHA ISOFORM

BCL2 Motifs	unique variants*	total mutations*	length of domain	frequency
N-term	20	42	9	4,7
BH4	**17**	**27**	**21**	**1,3**
FLD	64	120	62	1,9
BH3	**6**	**8**	**15**	**0,5**
between BH3 and BH1	19	34	28	1,2
BH1	**7**	**10**	**20**	**0,5**
between BH1 and BH2	10	11	31	0,4
BH2	**3**	**7**	**16**	**0,4**
C-terminus	0	0	38	0,0

* only non-synonymous mutations

Supplementary table 3

ALPHA isoform mutation	MA_score	MA_pred
A2T	1,845	low
H3Y	1,61	low
A4G	0	neutral
A4V	0	neutral
A4P	-1,7	neutral
A4T	0	neutral
G5E	1,39	low
R6T	1,39	low
R6K	1,39	low
T7M	0,345	neutral
T7I	0,895	low
T7A	0,895	low
T7P	1,245	low
T7R	-0,255	neutral
G8A	1,825	low
G8K	2,175	medium
G8R	2,175	medium
G8W	2,175	medium
Y9_D10insD		
Y9F	2,295	medium
Y9N	2,295	medium
D10E	1,87	low
N11Y	2,34	medium
N11T	1,995	medium
R12W	2,475	medium
E13D	1,355	low
I14L	0,975	low
M16V	0,2	neutral
M16L	0,35	neutral
K17R	1,78	low
H20Q	2,175	medium
H20R	1,825	low
S24A	2,475	medium
R26G	2,175	medium
R26K	0,68	neutral
R26T	2,175	medium
G27A	2,495	medium
E29A	1,355	low
D31E	0,235	neutral
D31G	1,04	low
A32G	1,845	low
A32T	1,845	low

G33R	2,215	medium
G33A	2,215	medium
G33V	2,215	medium
D34N	0,205	neutral
D34E	0,55	neutral
D34Y	0,55	neutral
G36D	-0,695	neutral
A37P	0,345	neutral
A42P	0,805	low
A42T	0,805	low
A42V	0,805	low
A45P	0,805	low
A45T	0	neutral
P46S	0,895	low
G47D	0,975	low
G47A	0,625	neutral
G47V	0,975	low
S50A	1,04	low
S50P	0,695	neutral
S51A	0	neutral
S51C	0,345	neutral
S51P	0,345	neutral
Q52H	0,975	low
Q52R	0,975	low
P53S	0,55	neutral
G54E	0	neutral
T56A	0,205	neutral
H58L	-0,345	neutral
P59A	0,975	low
P59S	0,625	neutral
P59L	0,625	neutral
A60V	0,975	low
A60G	0,625	neutral
A60S	0,625	neutral
A60D	0,975	low
A60T	0,975	low
A61T	0,695	neutral
S62F	0	neutral
S62T	0	neutral
P65S	0,205	neutral
V66A	-0,55	neutral
P71S	1,1	low
P71A	1,1	low
Q73E	0,345	neutral
Q73R	-0,345	neutral
T74A	0	neutral

T74I	0	neutral
T74S	-0,55	neutral
A76T	0,695	neutral
A80G	0,695	neutral
A80P	1,04	low
A82S	0,46	neutral
A82V	0,805	low
L86F	0,975	low
L86R	0,975	low
S87N	0,975	low
S87R	0,625	neutral
P90S	1,1	low
P90T	1,1	low
P91L	1,1	low
P91R	1,1	low
T96A	0,695	neutral
R98C	3,025	medium
G101_D102insDDFS		
G101V	2,755	medium
D102A	2,84	medium
F104L	0,07	neutral
Y108F	1,64	low
Y108H	1,525	low
Y108S	3,025	medium
A113V	0,895	low
A113G	0,44	neutral
S117R	-0,35	neutral
S117T	0,345	neutral
Q118R	2,225	medium
L119V	1,705	low
H120Y	1,48	low
F124C	0,69	neutral
F124L	0,69	neutral
F124V	0,69	neutral
R129H	2,42	medium
A131V	0,255	neutral
A131D	2,045	medium
A131T	0,695	neutral
V133M	3,095	medium
E135D	-0,57	neutral
E136D	2,39	medium
V142M	2,485	medium
N143T	2,59	medium
N143S	2,31	medium
F151L	1,435	low
F153I	3,265	medium

F153L	2,57	medium
M157L	-0,53	neutral
C158S	1,66	low
N163K	1,375	low
N163S	1,265	low
E165D	1,97	medium
E165G	0,555	neutral
E165K	2,08	medium
D171E	1,525	low
Y180F	1,51	low
H184Y	1,305	low
D191E	-0,485	neutral
D191N	1,15	low
G194D	3,355	medium

Supplementary table 4

	No mutation n=117	**Mutation n=135**	**p**
	n (%)	n (%)	
Baseline characteristics			
Age > 60 years	39 (33)	50 (37)	0,540
Male sex	61 (52)	66 (49)	0,607
Ann Arbor stage III/IV	103 (88)	127 (94)	0,128
ECOG PS ≥ 1	36 (31)	43 (32)	0,897
B symptoms present	28 (24)	38 (28)	0,453
BM involvement [1]	71 (63)	79 (60)	0,622
Elevated LDH [2]	31 (27)	46 (34)	0,168
Hemoglobin level < 12 g/dL	21 (18)	30 (22)	0,352
β2-microglobulin ≥ 3 mg/L [3]	33 (30)	46 (35)	0,376
Histological grade 3A or diffuse area	14 (12)	20 (15)	0,509
FLIPI score			
0-1 risk factors	30 (26)	20 (15)	
2 risk factors	40 (34)	53 (39)	0,099
3-5 risk factors	47 (40)	62 (46)	
Induction regimen			
R-CHOP	111 (95)	124 (92)	0,103
R-CVP	6 (5)	11 (8)	
Response to induction regimen [4]			
CR	46 (41)	52 (39)	
Cru	35 (31)	44 (33)	0,717
PR	29 (26)	35 (27)	
SD/PD	3 (3)	1 (1)	
Maintenance regimen			
Observation	51 (44)	74 (55)	0,168
Rituximab	50 (43)	50 (37)	
Not randomized in PRIMA trial	*16 (14)*	*11 (8)*	
IGH-BCL2 rearrangement	66 (56)	116 (86)	<0.001

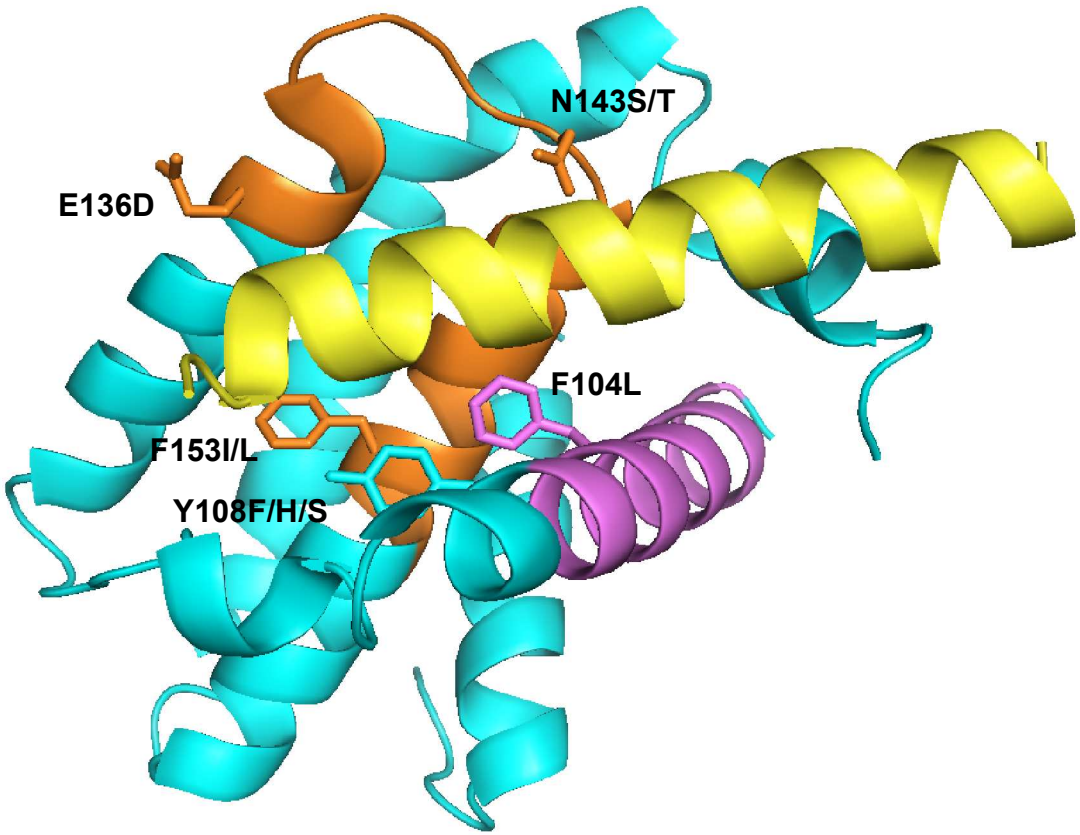
[1] BM involvement data were missing or not evaluated for 5 unmutated patient and 4 mutated patients, respectively.

[2] LDH data were missing for 1 unmutated patient.

[3] β2-microglobulin data were missing for 6 unmutated and 5 mutated patients, respectively.

[4] Response to induction regimen was missing or not evaluated for 4 unmutated patient and 3 mutated patients, respectively.

Supplemental Figure 1



Supplemental figure 2