MYD88 mutated and wild-type Waldenström's Macroglobulinemia: characterization of chromosome 6q gene losses and their mutual exclusivity with mutations in *CXCR4*

Waldenström's Macroglobulinemia (WM) is a lymphoplasmacytic lymphoma characterized by bone marrow (BM) infiltration of immunoglobulin M (IgM)-secreting lymphoplasmacytic cells.[1] Activating mutations in *MYD88* are present in 93-97% of WM and 50-70% of IgM monoclonal gammopathy of undetermined significance (MGUS) patients.[2] IgM MGUS patients with *MYD88* mutations may be at a higher risk of progression to WM.[3] Mutated MYD88 triggers WM cell growth and survival by activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pro-survival signaling through interleukin-1 receptor-associated kinase 1 (IRAK1)/IRAK4 and Bruton's tyrosine kinase (BTK). WM patients with wild-type *MYD88* (*MYD88*^{WT}) show recurring somatic mutations in *TBL1XR1, NFKB2*, and the CARD11-BCL10-MALT1 (CBM) complex genes *BCL10* and *MALT1*, and show shorter survival and a higher incidence of associated diffuse large B-cell lymphoma events *versus MYD88* mutated (*MYD88*^{MUT}) patients.[4] *MYD88*^{MUT} WM patients also show higher levels of response and progression-free survival to ibrutinib in comparison to *MYD88*^{WT} patients.[5] Activating *CXCR4* mutations are present in 30-40% of WM patients, and are typically subclonal to *MYD88* mutations.[6] *CXCR4* mutations trigger pro-survival protein kinase B (AKT) and extracellular signal-regulated kinase 1 (ERK1/2) signaling and are associated with inferior and/or delayed response to many WM therapeutics, including ibrutinib.[5,7] Deletions in chromosome 6q (del6q) occur in about 50% of WM patients, and are associated with the transition from IgM-MGUS to WM.[8,9] The functional role of del6q in this transition, and their relationship to *MYD88* and *CXCR4* mutations remain to be characterized. A minimal region of deletion (MDR) for 6q (6q14.1-6q27) in WM patients bearing the *MYD88* mutation was previously reported by us,[9] and included many genes with important regulatory functions for BTK/B-cell receptor (*IBTK*), apoptosis (*FOXO3*), (BCL2 *BCLAF1*) and NF-kB (*TNFAIP3, HIVEP2*), signaling (Figure 1A). In the study herein, we sought to delineate the gene losses related to del6q in asymptomatic and symptomatic WM, as defined by The Second International Workshop on WM (IWWM-2) criteria,[1] and their association to *MYD88* and *CXCR4* mutations and signaling.

The Dana-Farber/Harvard Cancer Center (DF/HCC) Institutional Review Board approved this study and the samples were collected following informed consent. The study cohort included 33 untreated WM patients (21 males, 12 females). Patients had a median age of 62 (range: 35-91) years, BM involvement of 60% (range: 2.5-90%), serum IgM levels of 3,010 (range: 257-6910) mg/dl, and hemoglobin of 10.9 (range: 8.4-14.4) g/dl. *MYD88* and *CXCR4* mutations were assessed using allele-specific polymerase chain reaction (PCR) and Sanger sequencing as previously described.[6] All *MYD88* mutations corresponded to p.Leu265Pro and were present in 25 (76%) patients, 11 (44%) of whom also carried *CXCR4* activating mutations (*CXCR4*^{MUT}). The eight asymptomatic WM patients were all *MYD88*^{MUT}, five of whom also carried *CXCR4*^{MUT}. The 25 symptomatic WM patients included 17 *MYD88*^{MUT} patients (six of whom were also *CXCR4*^{MUT}) and all eight *MYD88*^{WT}*CXCR4*^{WT} patients (Figure 1 B-D). For the five studied genes, copy number alterations (CNA) were measured in quadruplicate and gene expression in triplicate from CD19-selected BM lymphoplasmacytic cells with TaqMan real-time (RT) PCR protocols (Thermo Fisher Scientific, MA, USA)

Table 1. List of 19 genes found to be dysregulated in *MYD88*^{MUT} patients with chromosome 6q deletions or *CXCR4* mutations.

Gene	Chrom	Start	End	LogFC Del6q	LogFC CXCR4^{mut}
FAM110C	chr2	38813	41627	-3.61	-3.29
WNK2	chr9	95947211	95947892	-1.88	-4.92
SGCD	chr5	155135062	155135118	-1.87	-3.26
CCDC141	chr2	179694483	179699167	-1.67	-1.30
PKHD1L1	chr8	110374705	110374882	-1.56	-1.47
EML6	chr2	54952148	54952395	-1.49	-1.03
ZNF214	chr11	7020548	7022786	-1.39	-1.85
SYTL2	chr11	85405264	85406383	-1.32	-1.30
C11orf92	chr11	111164113	111169391	-1.31	-0.91
IL17RB	chr3	53880576	53880675	-1.26	-2.04
ZNF215	chr11	6947653	6947916	-1.19	-1.29
ZNF804A	chr2	185463092	185463797	-1.12	-0.69
LINC00271	chr6	135818938	135819138	-0.99	-0.69
CDK14	chr7	90095737	90095827	-0.76	-0.57
FOXO3	chr6	108881025	108881218	-0.75	-0.54
CYP4V2	chr4	187112673	187113191	-0.69	-0.53
IGF2R	chr6	160390130	160390427	-0.53	-0.43
EPS15	chr1	51819934	51822518	-0.39	-0.37
HRK	chr12	117299026	117299271	1.84	2.02

Based on next-generation RNA sequencing data previously published by Hunter *et al.*, the present list was derived from 131 genes affected by chromosome 6q deletions and 3103 genes associated with *CXCR4* mutations. Log-2 fold change (LogFC) estimates are shown relative to both 6q deletions and *CXCR4* mutations.

using the following assays for CNA and expression, respectively: *IBTK*: Hs01076984_cn/Hs00394118_m1; *FOXO3*: Hs01521732_cn/Hs00818121_m1; *BCLAF1*: Hs02855566_cn/Hs03004661_g1; *TNFAIP3*: Hs00548617_cn/Hs00234713_m1; *HIVEP2*: Hs01433181_cn/Hs00198801_m1. Paired CD19-depleted peripheral blood mononuclear cells (PBMC) were used as germline controls. Paired $CD19^+$ and $CD19^-$ PBMC from six healthy donors were included to rule out possible B-cell specific findings. Deletions affecting <20% of WM cells were considered to be under the RT-PCR detection threshold. Whole genome sequencing was previously performed in 17/33 (52%) patients and used to validate del6q TaqMan findings.[9] Previously published ribonucleic acid sequencing (RNASeq) data[10] were reanalyzed using Bioconductor in R (R Foundation for Statistical Computing, Vienna, Austria).

Comparing germline and tumor DNA by CNA assays revealed heterozygous somatic deletions for at least one 6q MDR gene evaluated in 20/25 (80%) $MYD88^{MUT}$ WM patients. No CNA for any of the 6q MDR genes were observed in any of the healthy donors. In $MYD88^{MUT}$ WM patients, *BCLAF1* was the most frequently deleted gene (19/25; 76%), followed by *TNFAIP3* (15/25; 60%). *HIVEP2, IBTK,* and *FOXO3* were each deleted in 13/25 (52%) of cases. Deletions for at least one 6q *MDR* gene were detected in 7/8 (88%) asymptomatic patients compared to 13/17 (76%; *P*=not significant [NS]) symptomatic $MYD88^{MUT}$ patients. Likewise, no individual gene demonstrated significantly different deletion rates between these groups. Our findings are therefore consistent with previous studies indicating that del6q was indicative of WM, regardless of symptomatic status.[8,11]

In $MYD88^{MUT}$ WM patients, two distinct patterns of del6q were identified. One group was comprised of 8/20 (40%) patients and showed more clonal and contiguous losses spanning across all MDR genes, while a second group (12/20; 60%) had more focal and subclonal genes

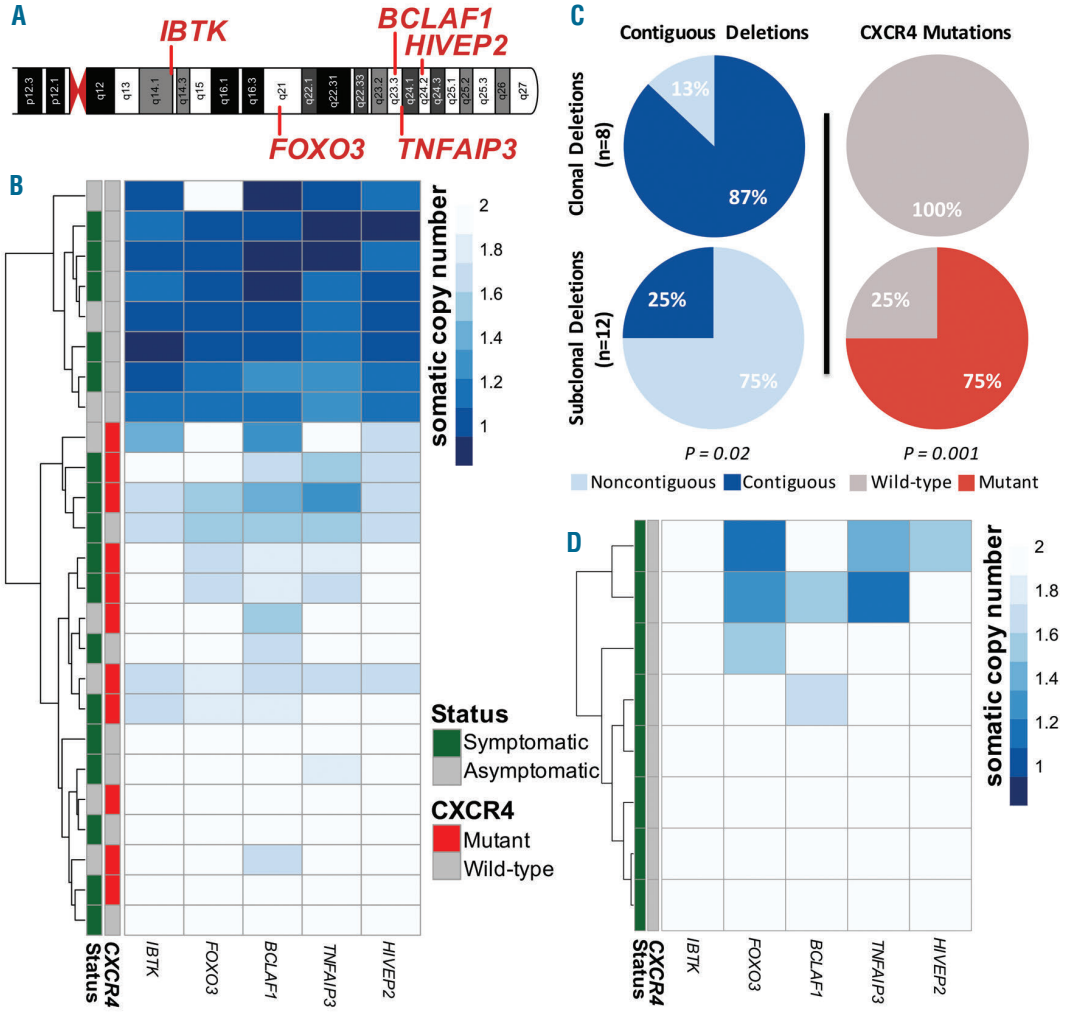

Figure 1. Characteristics of chromosome 6q deletions in Waldenström's Macroglobulinemia. (A) Ideogram of chromosome 6q showing the location of all study genes. (B) Heatmap of the statistically significant copy number alterations based on TaqMan RT-PCR analysis for $MYD88^{MUT}$ patients. Dark blue indicates a fully clonal deletion (copy number=1) for that gene. Hierarchical clustering of the patients revealed two distinct patterns of chromosome 6q deletion, whereby one group demonstrates more clonal and contiguous deletions, and the other more focal and subclonal gene losses. The mean deletion clonality per patient was highly statistically significant between the two groups (*P*=0.0002). No differences were observed based on symptomatic status. (C) Contiguous deletions and *CXCR4* mutation status stratified by the two populations (clonal and subclonal deleted) that were identified by hierarchical clustering in B. The analysis was restricted to patients with 6q deletions. (D) Heatmap of statistically significant copy number alterations based on TaqMan RT-PCR analysis for $MYD88^{WT}$ patients. No *CXCR4* mutations were detected in this population, and all patients had symptomatic disease.

losses (Figure 1B,C). The mean copy number estimate for deleted genes per patient was significantly lower in the eight patients with contiguous deletions (median 1.02; range: 0.98-1.20) compared with the other del6q patients (median 1.65; range: 1.43-1.75; *P*=0.0002). No differences in *MYD88*^{MUT} clonality were noted between the groups, ruling out normal B-cell contamination differences that might affect these findings. All eight of the clonal patients were *CXCR4*^{WT}, while *CXCR4* mutations were observed in 11/17 (65%) of the remaining patients (*P*=0.003). No other significant differences in the clinical features were noted. Contiguous del6q were not observed in the eight *MYD88*^{WT}*CXCR4*^{WT} patients. Non-contiguous deletions in the *MYD88*^{WT} cohort included *FOXO3, BCLAF1, TNFAIP3* and *HIVEP2* in three (38%), two (25%), two (25%) and one (13%), respectively, while *IBTK* remained intact (*P*=0.01 compared to *MYD88*^{MUT} WM, Figure 1D).

The number of patients in the *MYD88*^{MUT} cohort harboring at least one deleted gene was similar between the *CXCR4*^{MUT} (9/11; 82%) and *CXCR4*^{WT} (11/14; 79%; *P*=NS) populations. Because the nature of these deletions differed significantly with clonal contiguous deletions being mutually exclusive of *CXCR4* mutations, we used RT-PCR to investigate the effect of del6q and *CXCR4*^{MUT} on *CXCR4* transcript levels, and observed no differences in expression (*data not shown*). To investigate further, we performed principal component analysis on previously published RNASeq data from 57 WM patients using 131 genes that are differentially expressed in the presence of del6q.[10] This analysis not only stratified patients by del6q, but also by *MYD88/CXCR4* genotypes (Figure 2A) indicating that some of these genes are also modulated by *MYD88/CXCR4* status. The 20 most influential genes from the rotation matrix are available for the first two component *Online Supplementary Table S1*. Intersecting the gene lists associated with del6q and *CXCR4*^{MUT} revealed 19 overlapping genes, all of which change in the same direction in response to these somatic events (Table 1). As both

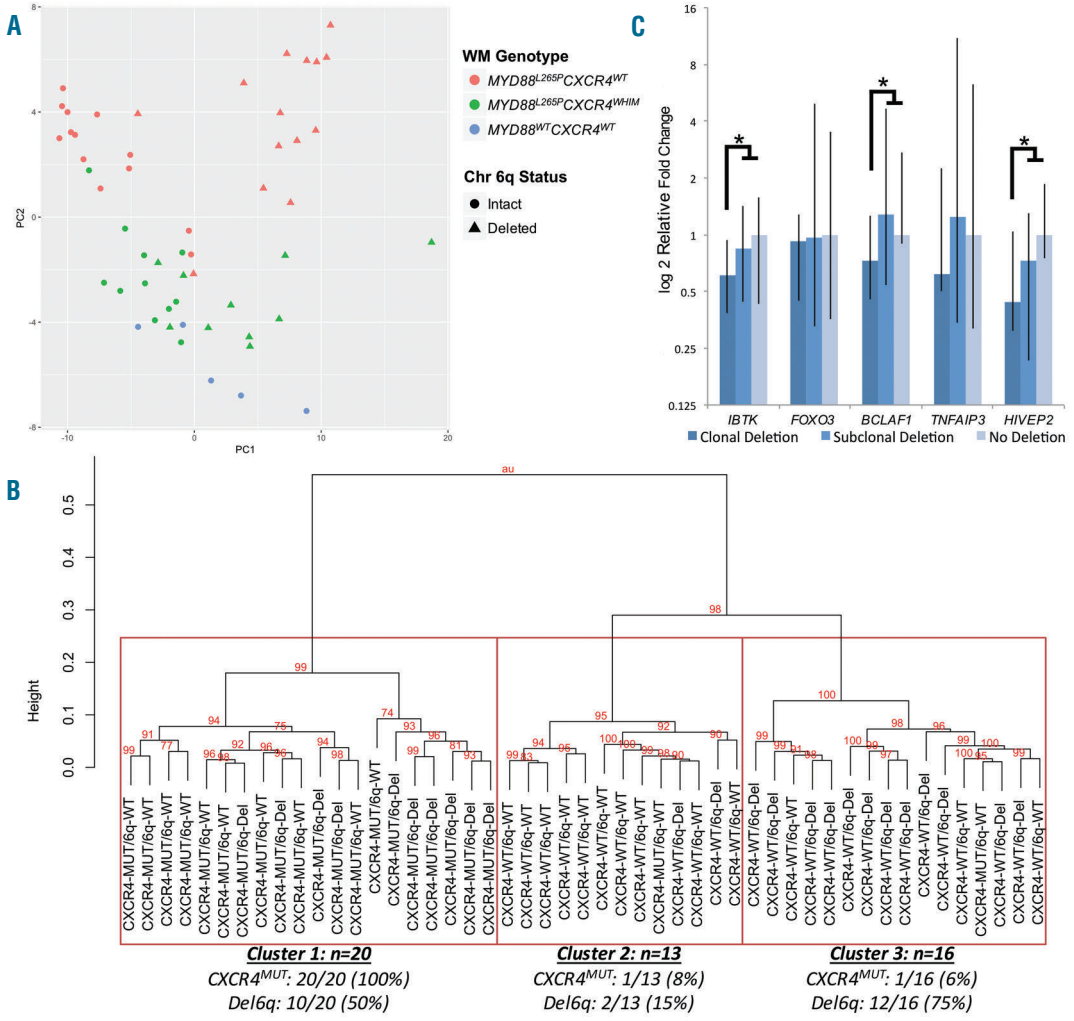

Figure 2. Transcriptional impact of chromosome 6q deletions in Waldenström's Macroglobulinemia. (A) Principal Component Analysis for 131 genes affected by 6q deletions based on next-generation RNA sequencing. Samples were stratified based on 6q deletion status on principal component 1 (PC1), and on *MYD88* and *CXCR4* genotype on principal component 2 (PC2). (B) Bootstrapped hierarchical clustering of the 19 genes that were similarly impacted by chromosome 6q deletions and *CXCR4* mutations in the *MYD88*^{MUT} RNASeq data. Approximate unbiased (AU) P-values are shown in red and represent the probability (%) of these samples clustering together under bootstrap simulations of similar populations. The three groups identified by this analysis stratified patients by 6q deletion and *CXCR4* mutation status (*P*<0.001 and *P*<0.005, respectively). (C) Real-time PCR gene expression results of samples from the *MYD88*^{MUT} cohort for each of the 6q groups identified. Relative fold change was calculated based on the median expression value for 6q intact patients. Median values and range are shown for each group. * indicates *P*-values <0.05 based on the presence of clonal deletions of that gene. WM: Waldenström's Macroglobulinemia; Chr: chromosome.

CXCR4^{MUT} and del6q are associated with the presence of *MYD88*^{MUT}, bootstrapped hierarchical clustering of the 19 genes was conducted on *MYD88*^{MUT} RNASeq data (Figure 2B). This generated three distinct clusters that significantly stratified patients based on del6q (P<0.005) and *CXCR4*^{MUT} (P<0.001) status. In context with previous studies that supported the acquisition of *CXCR4*^{MUT} and del6q after *MYD88*^{MUT},[6,8,11] these genes may play a critical role in WM clonal evolution.

Via quantitative RT-PCR, we sought to determine those study genes that were transcriptionally impacted by the presence of CNA in our study cohort. This analysis included all study samples and revealed that clonal deletions of *IBTK, BCLAF1* and *HIVEP2* significantly reduced the corresponding gene transcriptional levels in the eight clonally 6qdel *versus* all the other *MYD88*^{MUT} patients (P=0.03, P=0.01, P=0.01, respectively; Figure 2C).

Inhibitor of Bruton tyrosine kinase (IBTK) is a negative regulator of BTK, which is located downstream of mutated MYD88 and triggers pro-survival NF-κB signaling in WM.[12] The lack of *IBTK* deletions in *MYD88*^{WT} WM was notable as the BTK inhibitor ibrutinib shows poor activity in *MYD88*^{WT} WM patients, consistent with the notion that *BTK* is not essential for tumor survival in this patient population.[5] *BCLAF1* plays a pro-apoptotic role in the interaction with pro-survival BCL2 protein family members.[13] Its decreased expression may contribute to the survival of WM cells, which universally express high levels of BCL2.[10] *IBTK* and *BCLAF1* could potentially help delineate those patients who are suited for BTK-inhibitors and BCL-2 antagonist therapies. Moreover, the BCL-2 inhibitor venetoclax has shown significant activity in a phase I trial conducted in relapsed/refractory non-Hodgkin lymphoma patients, including WM,[14] and is currently under further investigation in relapsed/refractory WM. *HIVEP2*, which also showed decreased transcription levels in clonally deleted patients, blocks NF-κB nuclear signaling by binding to NF-κB consensus binding sites.[15] Surprisingly, *FOXO3* and *TNFAIP3*, which are important regulators of apoptosis and NF-κB signaling, respectively, were not impacted transcriptionally. Therefore, *IBTK, BCLAF1* and *HIVEP2* may serve as particularly important determinants of disease progression. However, the limited number of patients enrolled in the study herein precluded any investigation into the prognostic or predictive role for the 6q MDR genes examined, and further efforts into clarifying their clinical significance are warranted.

In *MYD88* mutated patients, fully clonal 6qdel and *CXCR4* mutation status showed mutual exclusivity, suggesting shared roles for the two genomic events. *CXCR4*^{MUT} was previously shown by us to downregulate tumor suppressors that are transcribed in response to mutated MYD88.[10] Indeed, herein we identified 19 genes co-regulated by 6qdel and *CXCR4* mutation status, which may be involved in WM clonal evolution. In summary, our findings provide new insights into WM pathogenesis, including loss of key regulators of BTK, apoptosis, BCL2 and NF-κB signaling in asymptomatic and symptomatic WM patients, and shared regulatory signaling for *MYD88*^{MUT} WM patients with either 6qdel or *CXCR4*^{MUT} disease.

Maria Luisa Guerrera,[1,2,5] Nickolas Tsakmaklis,[1] Lian Xu,[1] Guang Yang,[1,2] Maria Demos,[1] Amanda Kofides,[1] Gloria G. Chan,[1] Robert J. Manning,[1] Xia Liu,[1] Jiaji G. Chen,[1] Manit Munshi,[1] Christopher J. Patterson,[1] Jorge J. Castillo,[1,2] Toni Dubeau,[1] Joshua Gustine,[1] Ruben D. Carrasco,[3,4] Luca Arcaini,[5,6] Marzia Varettoni,[5] Mario Cazzola,[5,6] Steven P. Treon[1,2] and Zachary R. Hunter[1,2]

[1]Bing Center for Waldenström's Macroglobulinemia, Dana-Farber Cancer Institute, USA; [2]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; [3]Department of Oncologic Pathology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; [4]Department of Pathology, Brigham & Women's Hospital, Boston, MA, USA; [5]Department of Hematology and Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy and [6]Department of Molecular Medicine, University of Pavia, Italy

Funding: the authors acknowledge the contributions of the University of Pavia and the Fondazione Banca del Monte di Lombardia, Italy, the Leukemia and Lymphoma Society, Jon Orzag and Mary Kitchen Fund for Waldenstrom's Macroglobulinemia, and Peter S. Bing M.D. for supporting this work. ZRH is an ASH scholar.

Correspondence: zachary_hunter@dfci.harvard.edu

doi:10.3324/haematol.2018.190181

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Owen RG, Treon SP, Al-Katib A, et al. Clinicopathological definition of Waldenstrom's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenstrom's Macroglobulinemia. Semin Oncol. 2003;30(2):110-115.
2. Treon SP, Hunter ZR. A new era for Waldenstrom macroglobulinemia: MYD88 L265P. Blood. 2013;121(22):4434-4436.
3. Varettoni M, Zibellini S, Arcaini L, et al. MYD88 (L265P) mutation is an independent risk factor for progression in patients with IgM monoclonal gammopathy of undetermined significance. Blood. 2013; 122(13):2284-2285.
4. Treon SP, Gustine J, Xu L, et al. MYD88 wild-type Waldenstrom Macroglobulinaemia: differential diagnosis, risk of histological transformation, and overall survival. Br J Haematol. 2017;180(3):374-380.
5. Treon SP, Xu L, Hunter Z. MYD88 mutations and response to ibrutinib in Waldenström's Macroglobulinemia. N Engl J Med. 2015; 373(6):584-586.
6. Xu L, Hunter ZR, Tsakmaklis N, et al. Clonal architecture of CXCR4 WHIM-like mutations in Waldenström Macroglobulinaemia. Br J Haematol. 2015;172(5):735-744.
7. Cao Y, Hunter ZR, Liu X, et al. The WHIM-like CXCR4(S338X) somatic mutation activates AKT and ERK, and promotes resistance to ibrutinib and other agents used in the treatment of Waldenstrom's Macroglobulinemia. Leukemia. 2014;29(1):169-176.
8. Paiva B, Corchete LA, Vidriales M-B, et al. The cellular origin and malignant transformation of Waldenström's macroglobulinemia. Blood. 2015;125(15):2370-2380.
9. Hunter ZR, Xu L, Yang G, et al. The genomic landscape of Waldenström macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagenesis. Blood. 2014; 123(11):1637-1646.
10. Hunter ZR, Xu L, Yang G, et al. Transcriptome sequencing reveals a profile that corresponds to genomic variants in Waldenstrom macroglobulinemia. Blood. 2016;128(6):827-838.
11. Poulain S, Roumier C, Galiègue-Zouitina S, et al. Genome wide SNP array identified multiple mechanisms of genetic changes in Waldenstrom macroglobulinemia. Am J Hematol. 2013;88(11):948-954.
12. Liu W, Quinto I, Chen X, et al. Direct inhibition of Bruton's tyrosine kinase by IBtk, a Btk-binding protein. Nat Immunol. 2001;2(10):939-946.
13. Kasof GM, Goyal L, White E. Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. Mol Cell Biol. 1999;19(6):4390-4404.
14. Davids MS, Roberts AW, Seymour JF, et al. Phase I first-in-human study of venetoclax in patients with relapsed or refractory non-Hodgkin lymphoma. J Clin Oncol. 2017;35(8):826-833.
15. Iwashita Y, Fukuchi N, Waki M, Hayashi K, Tahira T. Genome-wide repression of NF-κB target genes by transcription factor MIBP1 and its modulation by O-linked β-N-acetylglucosamine (O-GlcNAc) transferase. J Biol Chem. 2012;287(13):9887-9900.