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MYD88 mutated and wild-type Waldenström's Macroglobulinemia: Characterization of chromosome 6q gene losses and their mutual exclusivity with mutations in _CXCR4_.

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Waldenström's Macroglobulinemia (WM) is a lymphoplasmacytic lymphoma characterized by bone marrow (BM) infiltration of IgM-secreting lymphoplasmacytic cells.[1] Activating mutations in *MYD88* are present in 93-97% of WM and 50-70% of IgM MGUS patients.[2] IgM MGUS patients with *MYD88* mutations may be at higher risk of progression to WM.[3] Mutated MYD88 triggers WM cell growth and survival by activation of NF-kB pro-survival signaling through IRAK1/IRAK4 and BTK. WM patients with wild-type *MYD88* (*MYD88*^{WT}) show recurring somatic mutations in *TBL1XR1*, *NFKB2*, and the 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 AKT and 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/BCR (*IBTK*), apoptosis (*FOXO3*), BCL2 (*BCLAF1*) and NF-kB (*TNFAIP3*, *HIVEP2*), signaling (**Figure 1A**). In this study, we sought to delineate the gene losses related to del6q in asymptomatic and symptomatic WM, as defined by IWWM-2 criteria[1], and their association to *MYD88* and *CXCR4* mutations and signaling.

The DF/HCC Institutional Review Board approved this study and the samples were collected following informed consent. Study cohort included 33 untreated WM patients (21 males, 12 females). Patients had median age of 62 (range 35-91) years, BM involvement of 60% (range 2.5-90%), serum IgM levels of 3,010 (range 257-6,910) mg/dl, and hemoglobin of 10.9 (range 8.4-14.4) g/dl. *MYD88* and *CXCR4* mutations were assessed using allele-specific 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 8 asymptomatic WM patients were all *MYD88*^{MUT}, 5 of whom also carried *CXCR4*^{MUT}. The 25 symptomatic WM patients included 17 *MYD88*^{MUT} *pa*tients (6 of whom were also *CXCR4*^{MUT}) and all 8 *MYD88*^{WT}*CXCR4*^{WT} patients (**Figure 1 B-D).** For the 5 studied genes, copy number

alterations (CNA) were measured in quadruplicate and gene expression in triplicate from CD19-selected BM lymphoplasmacytic cells with TaqMan RT-PCR protocols (Thermo Fisher Scientific, Waltham, MA) 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 6 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 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 compared to 13/17 (76%; p=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 losses (**Figure 1 B-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 8

MYD88^{WT}*CXCR4*^{WT} patients. Non-contiguous deletions in the *MYD88*^{WT} cohort included *FOXO3*, *BCLAF1*, *TNFAIP3* and *HIVEP2* in 3 (38%), 2 (25%), 2 (25%) and 1 (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 twenty most influential genes from the rotation matrix are available for the first two components in **Supplemental Table 1.** 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 *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 3 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.

By 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 8 clonally 6qdel versus all the other *MYD88*^{MUT} patients (p=0.03, 0.01, 0.01, respectively; **Figure 2C**).

IBTK is a negative regulator of BTK, which is downstream of mutated MYD88 and triggers pro-survival NF-kB 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 survival of WM cells, which universally express high levels of *BCL2*.[10] *IBTK* and *BCLAF1* could potentially help delineating 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-kB nuclear signaling by binding to NF-kB consensus binding sites.[15] Surprisingly, *FOXO3* and *TNFAIP3*, which are important regulators of apoptosis and NF-kB 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 on this study 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 down regulate tumor suppressors that are transcribed in response to mutated *MYD88*.[10] Indeed, here 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-kB signaling in asymptomatic and symptomatic WM patients, and shared regulatory signaling for $MYD88^{MUT}$ WM patients with either 6qdel or $CXCR4^{MUT}$ disease.

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Authorship

Contribution: M.L.G., Z.R.H. and S.P.T. designed the study and wrote the manuscript; M.L.G., N.T., M.D. and A.K. performed the RT-PCR assays on the samples; Z.R.H. and G.G.C. performed the data analysis and informatics studies; N.T., L.X., M.D., A.K., X.L., J.G.C. and M.M. prepared the study samples; S.P.T., J.J.C. and T.D. provided patient care and obtained consent and samples; M.L.G., R.J.M., J.G. and C.J.P. selected samples and provided clinical data analysis; S.P.T., Z.R.H., G.Y., M.C., M.V., L.A. and R.D.C. reviewed the data and provided expert guidance.

Conflict of Interest

The authors declare no competing financial interests.

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Tables

Table 1.

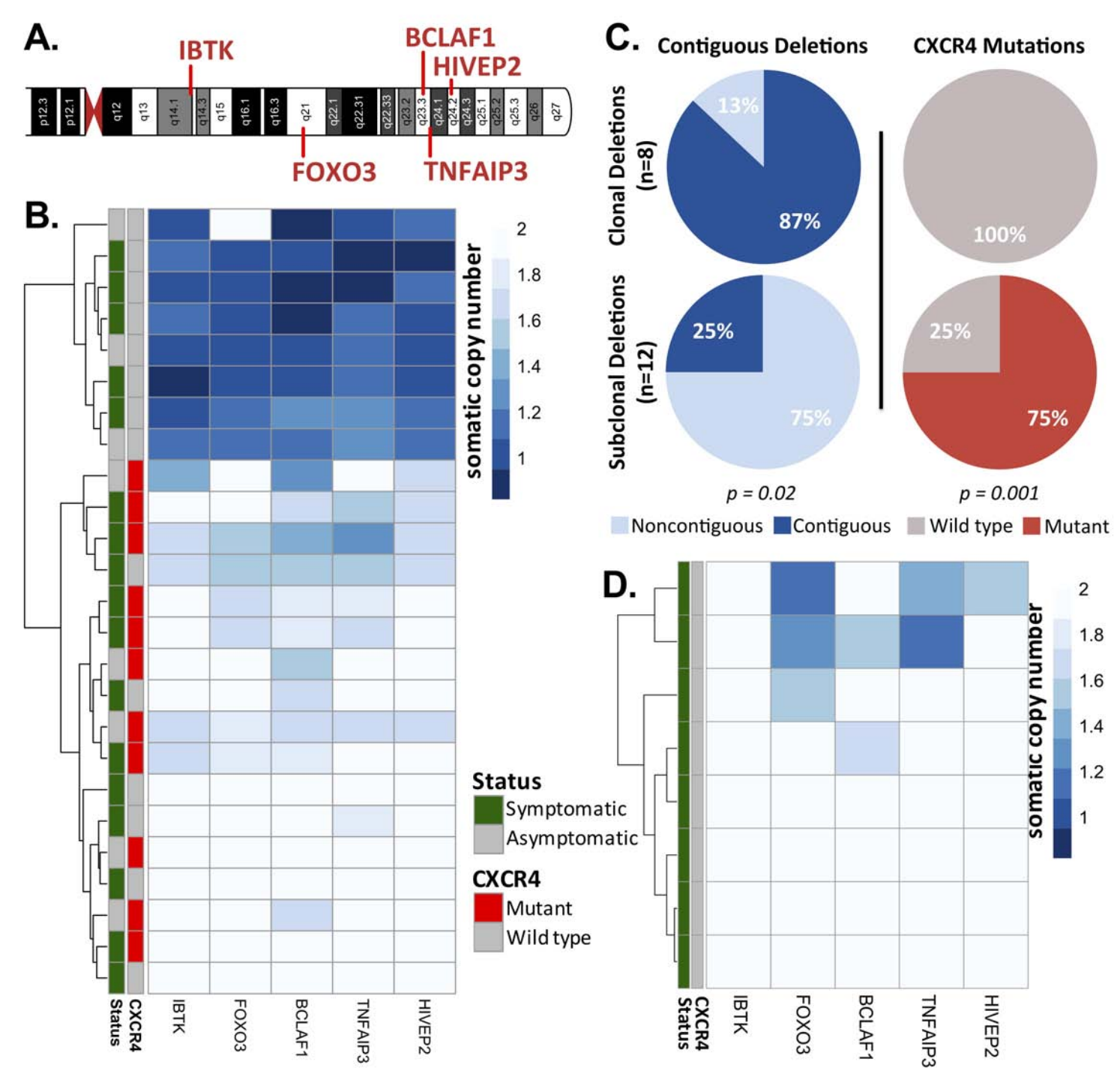
Gene	Chrom	Start	End	LogFC Del6q	LogFC CXCR4^{mut}
FAM110C	chr2	38813	41627	-3.61	-3.29
WNK2	chr9	95947211	95947892	-1.88	-4.92
SGCD	chr5	155135062	155135118	-1.87	-3.26
CCDC141	chr2	179694483	179699167	-1.67	-1.30
PKHD1L1	chr8	110374705	110374882	-1.56	-1.47
EML6	chr2	54952148	54952395	-1.49	-1.03
ZNF214	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

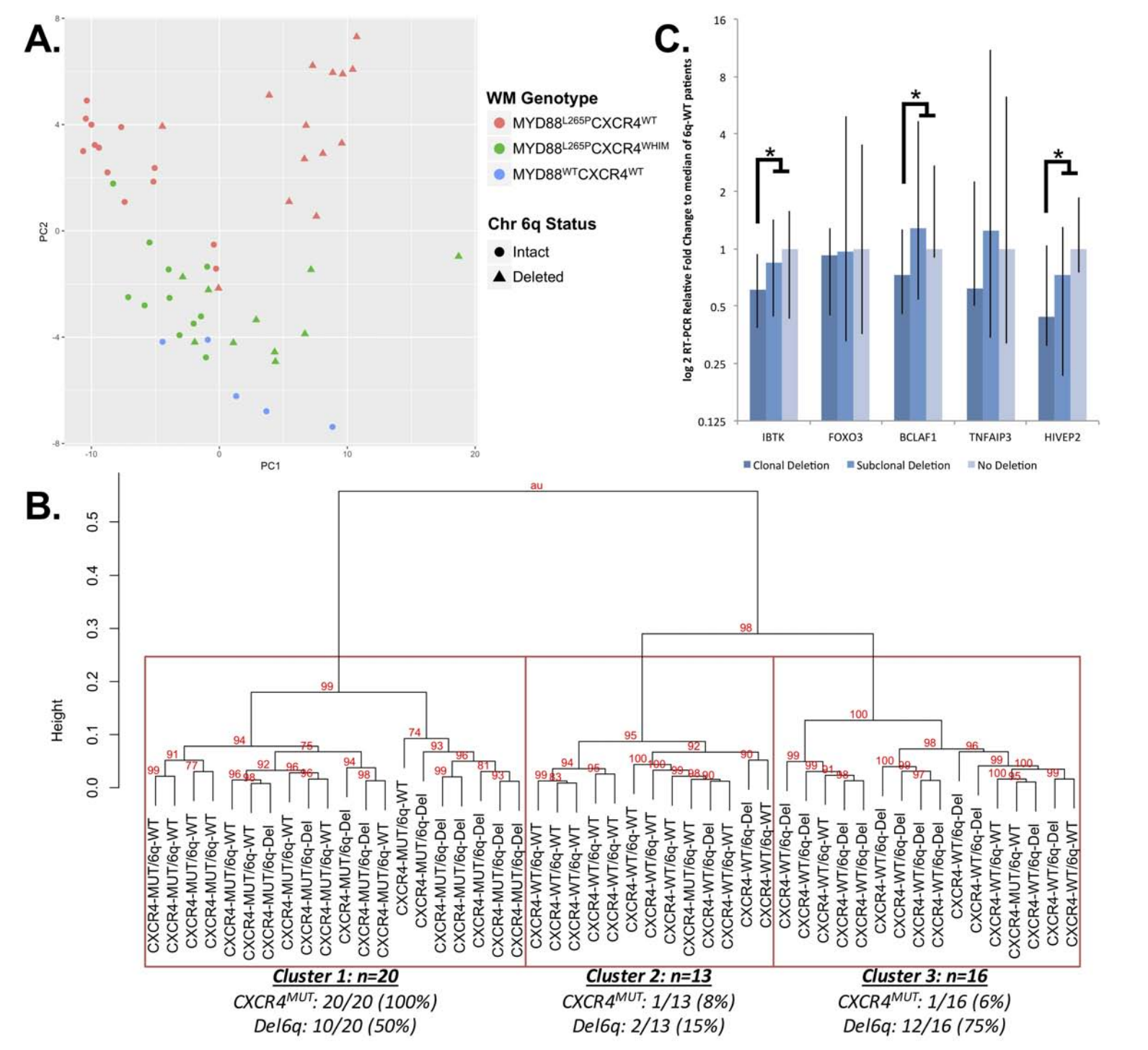
Table 1. **List of 19 genes found to be dysregulated in *MYD88^{MUT}* patients with chromosome 6q deletions or *CXCR4* mutations.** Based on next-generation RNA sequencing data previously published by Hunter et al., the present list was derived from 131 genes affected by chromosome 6q deletions and 3,103 genes associated with *CXCR4* mutations. Log-2 fold change (LogFC) estimates are shown relative to both 6q deletions and *CXCR4* mutations.

Figure Legends

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 with one group demonstrating 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.

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. The * indicates p-values <0.05 based on the presence of clonal deletions of that gene.


A.
IBTK
BCLAF1
HIVEP2
FOXO3
TNFAIP3
p12.3
p12.1
q12
q13
q14.1
q14.3
q15
q16.1
q16.3
q21
q22.1
q22.31
q22.33
q23.2
q23.3
q24.1
q24.2
q24.3
q25.1
q25.2
q25.3
q26
q27
B.
somatic copy number
2
1.8
1.6
1.4
1.2
1
Status
Symptomatic
Asymptomatic
CXCR4
Mutant
Wild type
Status
CXCR4
IBTK
FOXO3
BCLAF1
TNFAIP3
HIVEP2
C.
Contiguous Deletions
CXCR4 Mutations
Clonal Deletions (n=8)
Subclonal Deletions (n=12)
13%
87%
100%
25%
75%
25%
75%
p = 0.02
p = 0.001
Noncontiguous
Contiguous
Wild type
Mutant
D.
somatic copy number
2
1.8
1.6
1.4
1.2
1
Status
CXCR4
IBTK
FOXO3
BCLAF1
TNFAIP3
HIVEP2


A.
WM Genotype
MYD88L265PCXCR4WT
MYD88L265PCXCR4WHIM
MYD88WTCXCR4WT
Chr 6q Status
Intact
Deleted
PC1
PC2
C.
log 2 RT-PCR Relative Fold Change to median of 6q-WT patients
IBTK
FOXO3
BCLAF1
TNFAIP3
HIVEP2
Clonal Deletion
Subclonal Deletion
No Deletion
B.
Height
Cluster 1: n=20
CXCR4MUT: 20/20 (100%)
Del6q: 10/20 (50%)
Cluster 2: n=13
CXCR4MUT: 1/13 (8%)
Del6q: 2/13 (15%)
Cluster 3: n=16
CXCR4MUT: 1/16 (6%)
Del6q: 12/16 (75%)

Supplement

Supplemental Table 1.

Principal Component 1

	PC1	PC2
HIVEP2	-0.126	-0.038
STXBP5	-0.121	-0.083
NT5DC1	-0.117	-0.065
ASCC3	-0.115	-0.073
RSPH3	-0.112	0.023
C6orf120	-0.111	0.048
SENP6	-0.110	-0.040
LOC100507557	-0.109	0.077
LATS1	-0.109	-0.073
BC022047	-0.108	-0.082
MCM9	-0.107	-0.076
C6orf170	-0.107	-0.012
HINT3	-0.106	-0.068
BCLAF1	-0.106	-0.013
PPIL4	-0.105	-0.024
SERPINB9	-0.105	0.027
DSE	-0.105	-0.076
TULP4	-0.105	0.055
DQ599242	-0.104	-0.005
H1F0	0.104	0.063

Principal Component 2

	PC1	PC2
WNK2	-0.034	0.217
IL17RB	-0.046	0.199
SGCD	-0.053	0.183
ZNF214	-0.071	0.180
ZNF215	-0.072	0.173
ZNF804A	-0.076	0.159
EML6	-0.081	0.154
ECHDC1	-0.054	-0.152
LACE1	-0.096	-0.151
ORC3	-0.088	-0.144
HRK	0.075	-0.137
ZBTB24	-0.085	-0.135
BC071802	-0.054	0.133
QRSL1	-0.075	-0.132
CYP4V2	-0.065	0.130
FAM110C	-0.073	0.128
SYTL3	-0.100	0.126
LOC285758	-0.088	0.125
MACC1	-0.085	0.123
PKHD1L1	-0.071	0.119

Supplemental Table 1: Top 20 most influential genes for principal components 1 and 2 from the principal component analysis of genes associated with chromosome 6q deletions.