MicroRNA and long non-coding RNA analysis in IgM-monoclonal gammopathies reveals epigenetic influence on cellular functions and oncogenesis

Authors

Karan Chohan,[1*] Jonas Paludo,[2*] Surendra Dasari,[3] Patrizia Mondello,[2] Joseph P. Novak,[2] Jithma P. Abeykoon,[2] Kerstin Wenzl,[2] Zhi-Zhang Yang,[2] Shahrzad Jalali,[2] Jordan E. Krull,[2] Esteban Braggio,[4] Michelle K. Manske,[2] Aneel Paulus,[5] Craig B. Reeder,[4] Sikander Ailawadhi,[5] Asher Chanan-Khan,[5] Prashant Kapoor,[2] Robert A. Kyle,[2] Morie A. Gertz,[2] Anne J. Novak[2] and Stephen M. Ansell[2]

[1]Department of Medicine, Mayo Clinic, Rochester, MN; [2]Division of Hematology, Mayo Clinic, Rochester, MN; [3]Department of Health Sciences Research, Mayo Clinic, Rochester, MN; [4]Division of Hematology and Medical Oncology, Mayo Clinic, Scottsdale, AZ and [5]Division of Hematology and Oncology, Mayo Clinic, Jacksonville, FL, USA

**KC and JP contributed equally as first authors.*

Correspondence:
S.M. ANSELL - ansell.stephen@mayo.edu

https://doi.org/10.3324/haematol.2023.283927

Received: July 13, 2023.
Accepted: November 27, 2023.
Early view: December 7, 2023.

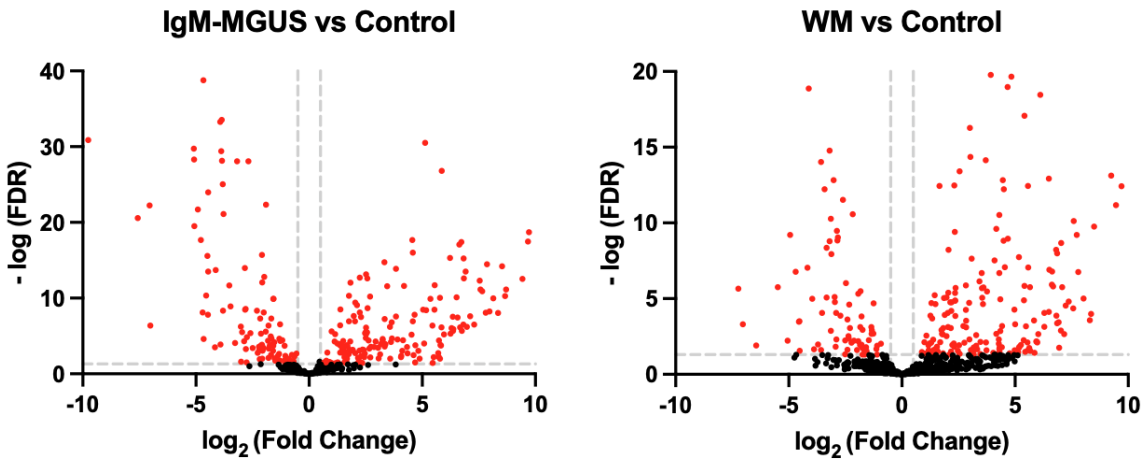
Supplemental Material

MicroRNA and long non-coding RNA analysis in IgM monoclonal gammopathies reveals epigenetic influence on cellular functions and oncogenesis

Karan Chohan[1#], Jonas Paludo[2#], Surendra Dasari[3], Patrizia Mondello[2], Joseph P Novak[2], Jithma P. Abeykoon[2], Kerstin Wenzl[2], Zhi-Zhang Yang[2], Shahrzad Jalali[2], Jordan E Krull[2], Esteban Braggio[4], Michelle K. Manske[2], Aneel Paulus[5], Craig B. Reeder[4], Sikander Ailawadhi[5], Asher Chanan-Khan[5], Prashant Kapoor[2], Robert A. Kyle[2], Morie A Gertz[2], Anne J. Novak[2] and Stephen M. Ansell[2]

[1]Department of Medicine, Mayo Clinic, Rochester, MN.
[2]Division of Hematology, Mayo Clinic, Rochester, MN
[3]Department of Health Sciences Research, Mayo Clinic, Rochester, MN
[4]Division of Hematology and Medical Oncology, Mayo Clinic, Scottsdale, AZ
[5]Division of Hematology and Oncology, Mayo Clinic, Jacksonville, FL
#Authors contributed equally

Supplemental Figure 1: Comparison of miRNA expression in IgM-MGUS compared to controls (A) and WM compared to controls (B). Dotted lines on volcano plot indicate significance thresholds for fold change and false discovery rate (FDR). Red markers indicate differentially expressed miRNA. Black markers indicate non-differentially expressed miRNA. Created using GraphPad Prism.



Supplemental Table 1: Pathways of interest between comparison groups and significant findings with underlying mRNA and miRNA expression profile.

Pathway of Interest	Comparison Group	Significant Findings
Cell Signaling	**IgM-Gammopathies to Normal Controls**	Upregulation of multiple signaling pathways, including STAT3, PI3K/AKT, JAK2 and RAC was observed in IgM-gammopathies compared to controls. Several of these pathways have been previously implicated in WM pathogenesis.[1,2] Upregulation of the *STAT3* gene was found to be commonly dysregulated in multiple pathways, which is a predicted target of multiple downregulated miRNAs, including miR-20B and miR-223. Previously, miRNA-223 downregulation has been implicated in a positive feedback loop to induce IL-6 and IL-1B production through targeting of *STAT3* in macrophages.[3] Additional genes implicated in these cell signaling pathways included, *BCL2* which was observed to be upregulated, and potentially epigenetically targeted by miR-16, miR-17, miR-181 and miR-34, all downregulated. Of note, *BCL2* is a regulator of mitochondrial apoptotic pathways and is a critical gene in WM pathogenesis induced through signaling of the *MYD88* and *CXCR4* activating mutations.[4,5]
	WM to IgM-MGUS	Multiple miRNA-based cell signaling pathways were observed to be differentially activated comparing WM to IgM-MGUS. Importantly, PTEN signaling was found to be activated in WM. The PI3K/AKT/mTOR pathway and the phosphate and tensin homolog tumor suppressor (PTEN) protein have been found to be crucial in cancer pathogenesis and cell proliferation.[2] Additionally, AKT has been demonstrated to be constitutionally activated in WM.[2,6] One of the implicated genes underlying the PTEN pathway was *FGFR3* which has been demonstrated to lead to induction of proliferation in WM. Comparing WM to IgM-MGUS, we found *FGFR3* to be downregulated in WM and a predicted target of the upregulated miRNA-194.[7]
Proliferation, and Death Regulation	**IgM-Gammopathies to Normal Controls**	Pathways involved in proliferation and death regulation were dysregulated between IgM-gammopathies and normal controls and between WM and IgM-MGUS. In IgM-gammopathies compared to controls, apoptosis-related pathways were found to be downregulated. Underlying multiple pathways was decreased expression of *BCL2* and *STAT3*, targeted by multiple miRNAs as specified above. In addition, there was upregulation of *FAS*, which codes for the Fas cell surface death receptor (FAS), a death receptor implicated in apoptosis.[8] *FAS* was found to be targeted experimentally by the downregulated miR-21-5p, which has previously been demonstrated to be involved in apoptosis through PTEN/PI3K/AKT signaling.[9] The ferroptosis pathway, an iron-dependent programmed cell death, was additionally found to be differentially activated when comparing IgM-gammopathies to controls.[10] We additionally observed increased expression of the *GPX4* gene in IgM-gammopathies, which is targeted by miR-1260a observed to be downregulated. Previously, our group has demonstrated that increased expression of GPX4 in WM plays a critical role in ferroptosis by promoting cell survival and inhibiting apoptosis.[11]

	WM to IgM-MGUS	Comparing WM to IgM-MGUS, alteration in inhibitor of DNA binding (ID)-1 and high mobility group box 1 (HMGB1) signaling pathways were observed. The ID proteins are involved in the regulation of cell-cycle progression and differentiation.[12] Underlying the ID1 pathway, we observed downregulation of B-cell lymphoma 3 (*BCL3*) which is a target of miR-19b, observed to be upregulated. *BCL3* regulates tumor development and progression and has been implicated in a number of malignancies.[13] HMGB1 is a non-histone chromosome-binding protein that interacts with DNA to regulate transcription, replication and repair, but is also secreted where it can act as a cytokine.[14] Among the altered genes in this pathway, *MAP2K3* was found to be downregulated and targeted by the upregulated miR-19b. Mitogen-activated protein kinase 3 (*MAP2K3*) is a member of the MAPK family, which are regulators of cell proliferation and have been found to be involved in WM pathogenesis.[6,15] Other genes in the MAPK family genes found to be upregulated included *MAP4K1*, which is a target of miR-143, observed to be downregulated. This implicates miRNAs as potential regulators of multiple MAPK genes in WM compared to IgM-MGUS, and more broadly the PI3K/AKT/mTOR pathway which plays a key role in cellular proliferation.
Cell Cycle Regulation: Alteration of Cyclin-Dependent Kinases	**IgM-Gammopathies to Normal Controls**	Comparing IgM-gammopathies to normal controls, significant alteration of cell cycle regulation pathways was observed. This included multiple checkpoint control pathways, as well as pathways involved in chromosomal replication. Underlying these pathways was the alteration of multiple cyclin-dependent kinase (CDK) genes, including *CDK1*, *CDK6*, *CDKN1A* and *CDKN2B*. Here, *CDK1* and *CDK6* were found to be downregulated, while *CDKN1A* and *CDKN2B* were upregulated. The differentially expressed miRNA (mRNA in bracket) which targeted these CDK family genes included let-7D (*CDK1*), miR-24 (*CDK1*), miR-30e (*CDK6*), all of which were upregulated, and miR-32 (*CDKN1A*), miR-590 (*CDKN1A*), miR-20a (*CDKN2B*), all which were downregulated. The CDK family are protein kinases that regulate cell cycle progression.[16] CDK inhibitor 1A (CDKN1A) and CDK inhibitor 2B (CDKN2B) are both inhibitors of cyclin and CDK complexes. Thus, we observed a miRNA-based inhibition mechanism of CDK family genes in IgM-gammopathies.
Metabolism	**IgM-Gammopathies to Normal Controls**	In IgM-gammopathies compared to controls, alteration in the calpain protease and HIF1a regulation pathways involved in metabolism was observed. Underlying these pathways included the downregulation of *PIK3C2B*, found to be experimentally targeted by miR-30b and miR-486, both upregulated. Additionally, we observed upregulation of *PIK3R2*, targeted by miR-34a and miR-491, both downregulated. *PIK3C2B* and *PIK3R2* are members of the phosphoinositide 3-kinase (PI3K) family, indicating the potential influence of miRNAs underlying this pathway in IgM-gammopathies. PI3K family proteins are demonstrated to regulate multiple facets of cancer development and metabolism through regulation of nutrient transporters and metabolic enzymes.[17]

Cell Structure	**IgM-Gammopathies to Normal Controls**	Alteration was additionally seen in multiple cytoskeleton pathways (integrin, actin regulation and paxillin signaling) when comparing IgM-gammopathies to controls. Multiple pathways had involvement of *PIK3C2B*, *ITGB2, ITGAL*, *MRAS*, and *RRAS,* all of which were upregulated. Underlying these mRNAs, we observed targeting miRNA with corresponding downregulation, including miR-34a which targets both *MRAS* and *RRAS*, miR-17 a target of *ITGAL* and miR-378 a target of *ITGB2*. These genes have previously been demonstrated to have an important function in the cytoskeleton, cell-cell structure, and the TME. Integrin beta 8 (ITBG8) belongs to the integrin beta chain family and plays a role in the integrin complex development which mediates the cell-cell and cell-extracellular matrix (ECM). ITBG8 has also been previously associated with glioblastoma angiogenesis and tumor cell invasiveness.[18] Integrin alpha L (ITGAL), interacts with ITGB2 to form the integrin lymphocyte function-associated antigen-1 (LFA-1) which is involved in intercellular adhesion and has previously been demonstrated to be involved in WM.[19] M-RAS (Ras-related protein) participate in actin cytoskeleton reorganization and through induction of AKT, has been shown to be associated with cell survival.[20] R-RAS, another RAS-related protein, has been demonstrated to associate with BCL-2.[21]
Cytokine Signaling Pathways	**WM to IgM-MGUS**	MiRNA-based inactivation of multiple inflammatory and cytokine signaling pathways in WM as compared to IgM-MGUS was observed. A pathway of particular interest was interferon signaling. Interferons are cytokines that have important roles in the regulation of biological processes, inflammation and tumorigenesis.[22] Underlying the interferon pathway was downregulation of multiple interferon-induced genes (*IFIT1, IFIT3, IFITM1*) and *STAT1*. The most notable targets of these interferon-induced genes were miR-32 (*IFIT2*, *IFITM1*), miR-146 (*IFIT2*, *IFIT3*) and miR-155 (*IFIT1*), all of which were upregulated. Additionally, underlying multiple inflammatory signaling and cytokine pathways was upregulation of miRs-146a, 150, 194, all targets of *STAT1*, observed to be downregulated. Of relevance, miR-146a has been previously shown to be an inhibitor of inflammatory cytokines, including IL-6.[23] Signal Transducer and Activator of Transcription (STAT) family proteins play a crucial role in the transmission of interferon-stimulated genes and cytokine signal transduction.[22] Downregulation of multiple cytokines signaling pathways involved in inflammation and the TME, along with the targeting of the STAT family, indicates a possible miR-based negative feedback loop to down-modulate inflammatory cytokine signaling in WM compared to IgM-MGUS.

Alteration of the Tumor Microenvironment	**WM to IgM-MGUS**	In WM compared to IgM-MGUS, there was a significant alteration of MMPs and FGF/FGFR family genes observed. Previously, MMPs have been demonstrated to be important in tumor cell proliferation.[24] They function to degrade components of the ECM and have been implicated in WM pathogenesis.[19,25] *MMP2*, *MMP8*, *MMP19* and *MMP25*, were found to be downregulated, while *MMP11* was found to be upregulated. Underlying these MMP genes was implication of multiple miRNAs (predicted target) including let-7e (*MMP11*) and miR-125a (*MMP11*) which were downregulated, and miR-296 (*MMP8*), miR-6803 (*MMP25*) and miR-20a (*MMP2*) which were upregulated. Conversely, the FGF/FGFR family serves an important role in the structural regulation of the TME.[26] In relation to the FGF/FGFR family genes, we observed several members to be potentially epigenetically regulated by miRNAs. *FGF13*, *FGF7* and *FGFR3* were all downregulated, predicted to be targeted by miR-155 (*FGF7* and *FGF13*), miR-16 (*FGF7*) and miR-194 (*FGFR3*), all which were upregulated. Previous investigations have demonstrated the role of miR-155 in the regulation of the FGF axis in a number of diseases.[27] These findings indicate the potential miRNA-based influence on the MMP and FGF/FGRF families and an epigenetic mechanism for regulating the TME structure in WM.

Supplemental Table 2: LncRNA analysis with previously published lncRNA and antisense mRNA analysis

lncRNA Analysis	Significant Findings
Oncologic lncRNA	lncRNAs that have been previously published and found to be differentially expressed in our analysis included LINC00943, ENSG00000274987 and ENSG00000237481.[28-30] LINC00943 has been demonstrated to regulate gastric cancer proliferation where it is aberrantly overexpressed and acts via regulation of miR-101-3p.[29] In our analysis, we found LINC00943 to be upregulated in WM, and additionally observed miR-101-3p (FC: 1.36; FDR: 0.06) to be upregulated in WM compared to IgM-MGUS, with this finding approaching significance. In patients with multiple myeloma (MM), lncRNA expression profiles from a database of 559 patients revealed four lncRNA were correlated with overall survival, including ENSG00000237481.[30] We additionally observed ENSG00000237481 to be upregulated in patients with WM as compared to IgM-MGUS. Lastly, in an analysis of patients with lung adenocarcinoma, assessment of immune-related lncRNAs revealed seven novel lncRNAs were predictive of prognosis in patients, including ENSG00000274987.[28] In our analysis, we similarly found this lncRNA to be differentially upregulated in WM compared to IgM-MGUS.
Antisense and Nearest Coding mRNA Analysis: Potential Role of lncRNA in Transcription Regulation and apoptosis	Several lncRNAs were found to be in proximity or antisense to genes responsible for transcription and cell cycle regulation. This included ENSG00000274987 found to be upregulated, which is the antisense lncRNA to *KRAS*. *KRAS* codes for the protein K-RAS, a GTPase implicated in B-cell proliferation and cell signaling, that is commonly dysregulated in lymphoid malignancies.[31,32] MSTRG.11369 was found to be downregulated, which is the antisense lncRNA to the gene *RASSF6* which has previously been demonstrated to be downregulated in WM*. RASSF6* codes for Ras-associated domain family member 6 (RASSF6) which is involved in cell cycle regulation and acts as a tumor suppressor.[33] Additionally, MSTRG.10284 was found to be downregulated, with *SATB1* as the closest protein-coding gene. *SATB1* codes for special AT-rich sequence-binding protein-1 (STATB1), a chromatin organizer and transcription factor which has been previously found to be downregulated in WM.[34] Next, assessing lncRNA with a nearest or antisense gene also differentially expressed on our mRNA analysis between WM and IgM-MGUS several targets of interest were found. Here, upregulation of ENSG00000229418 was observed, which is the antisense lncRNA to the gene *DNTT*, observed to be highly downregulated in WM (FC: -3.6; FDR: <0.005) compared to IgM-MGUS. DNA nucleotidylexotransferase (DNTT) is a DNA polymerase expressed in immature B and T lymphoid cells and previously has been found to be implicated in WM in relation to V(D)J recombination.[35] MSTRG.13296 is a lncRNA that was observed to be downregulated. The nearest gene to MSTRG.13296 is *BACH2*, which was significantly downregulated in WM (FC: -2.9; FDR <0.005) compared to IgM-MGUS. BACH2 is a B-cell transcription factor and tumor suppressor that has been demonstrated to be associated with an indolent clinical presentation in WM.[36] We additionally observed the downregulation of MSTRG.3833. The closest gene to MSTRG.3833 is *HRK* (FC: -2.4; FDR: <0.005) which was observed to be downregulated in WM compared to IgM-MGUS. *HRK* codes for the activator of apoptosis harakiri, which interacts with BCL-2 to regulate apoptosis, and has been previously demonstrated to be dysregulated in WM.[37,38]

References

1. Hodge LS, Ansell SM. Jak/Stat pathway in Waldenstrom's macroglobulinemia. *Clin Lymphoma Myeloma Leuk.* 2011;11(1):112-114.
2. Sacco A, Roccaro A, Ghobrial IM. Role of dual PI3/Akt and mTOR inhibition in Waldenstrom's Macroglobulinemia. *Oncotarget.* 2010;1(7):578-582.
3. Chen Q, Wang H, Liu Y, et al. Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1beta production in macrophages by targeting STAT3. *PLoS One.* 2012;7(8):e42971.
4. Kapoor I, Bodo J, Hill BT, Hsi ED, Almasan A. Targeting BCL-2 in B-cell malignancies and overcoming therapeutic resistance. *Cell Death Dis.* 2020;11(11):941.
5. Gaudette BT, Dwivedi B, Chitta KS, et al. Low expression of pro-apoptotic Bcl-2 family proteins sets the apoptotic threshold in Waldenstrom macroglobulinemia. *Oncogene.* 2016;35(4):479-490.
6. Leleu X, Jia X, Runnels J, et al. The Akt pathway regulates survival and homing in Waldenstrom macroglobulinemia. *Blood.* 2007;110(13):4417-4426.
7. Azab AK, Azab F, Quang P, et al. FGFR3 is overexpressed waldenstrom macroglobulinemia and its inhibition by Dovitinib induces apoptosis and overcomes stroma-induced proliferation. *Clin Cancer Res.* 2011;17(13):4389-4399.
8. Strasser A, Jost PJ, Nagata S. The many roles of FAS receptor signaling in the immune system. *Immunity.* 2009;30(2):180-192.
9. Buscaglia LE, Li Y. Apoptosis and the target genes of microRNA-21. *Chin J Cancer.* 2011;30(6):371-380.
10. Zhang C, Liu X, Jin S, Chen Y, Guo R. Ferroptosis in cancer therapy: a novel approach to reversing drug resistance. *Mol Cancer.* 2022;21(1):47.
11. Jalali S, Shi J, Ahsan N, et al. Progression from Monoclonal gammopathy of undetermined significance of the immunoglobulin M class (IgM-MGUS) to Waldenstrom Macroglobulinemia is associated with an alteration in lipid metabolism. *Redox Biol.* 2021;41:101927.
12. Roschger C, Cabrele C. The Id-protein family in developmental and cancer-associated pathways. *Cell Commun Signal.* 2017;15(1):7.
13. Niu Y, Yang X, Chen Y, et al. BCL3 Expression Is a Potential Prognostic and Predictive Biomarker in Acute Myeloid Leukemia of FAB Subtype M2. *Pathol Oncol Res.* 2019;25(2):541-548.
14. Yuan S, Liu Z, Xu Z, Liu J, Zhang J. High mobility group box 1 (HMGB1): a pivotal regulator of hematopoietic malignancies. *J Hematol Oncol.* 2020;13(1):91.
15. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res.* 2002;12(1):9-18.
16. Malumbres M. Cyclin-dependent kinases. *Genome Biol.* 2014;15(6):122.
17. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov.* 2009;8(8):627-644.
18. Tchaicha JH, Reyes SB, Shin J, Hossain MG, Lang FF, McCarty JH. Glioblastoma angiogenesis and tumor cell invasiveness are differentially regulated by beta8 integrin. *Cancer Res.* 2011;71(20):6371-6381.
19. Ghobrial IM, Zhang Y, Liu Y, et al. Targeting the bone marrow in Waldenstrom macroglobulinemia. *Clin Lymphoma Myeloma Leuk.* 2011;11 Suppl 1(Suppl 1):S65-69.
20. Matsumoto K, Asano T, Endo T. Novel small GTPase M-Ras participates in reorganization of actin cytoskeleton. *Oncogene.* 1997;15(20):2409-2417.
21. Fernandez-Sarabia MJ, Bischoff JR. Bcl-2 associates with the ras-related protein R-ras p23. *Nature.* 1993;366(6452):274-275.

22. Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol.* 2014;32:513-545.
23. Zhou C, Zhao L, Wang K, et al. MicroRNA-146a inhibits NF-kappaB activation and pro-inflammatory cytokine production by regulating IRAK1 expression in THP-1 cells. *Exp Ther Med.* 2019;18(4):3078-3084.
24. Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J.* 1999;13(8):781-792.
25. Zhou Y, Liu X, Xu L, et al. Matrix metalloproteinase-8 is overexpressed in Waldenstrom's macroglobulinemia cells, and specific inhibition of this metalloproteinase blocks release of soluble CD27. *Clin Lymphoma Myeloma Leuk.* 2011;11(1):172-175.
26. Sacco A, Federico C, Giacomini A, et al. Halting the FGF/FGFR axis leads to antitumor activity in Waldenstrom macroglobulinemia by silencing MYD88. *Blood.* 2021;137(18):2495-2508.
27. Moura J, Sorensen A, Leal EC, et al. microRNA-155 inhibition restores Fibroblast Growth Factor 7 expression in diabetic skin and decreases wound inflammation. *Sci Rep.* 2019;9(1):5836.
28. Jin D, Song Y, Chen Y, Zhang P. Identification of a Seven-lncRNA Immune Risk Signature and Construction of a Predictive Nomogram for Lung Adenocarcinoma. *Biomed Res Int.* 2020;2020:7929132.
29. Xu Y, Ji T, An N, Wang X, Zhang H, Xu F. LINC00943 is correlated with gastric cancer and regulates cancer cell proliferation and chemosensitivity via hsa-miR-101-3p. *Int J Clin Oncol.* 2021;26(9):1650-1660.
30. Zhou M, Zhao H, Wang Z, et al. Identification and validation of potential prognostic lncRNA biomarkers for predicting survival in patients with multiple myeloma. *J Exp Clin Cancer Res.* 2015;34(1):102.
31. Vendramini E, Bomben R, Pozzo F, et al. KRAS and RAS-MAPK Pathway Deregulation in Mature B Cell Lymphoproliferative Disorders. *Cancers (Basel).* 2022;14(3).
32. Jancik S, Drabek J, Radzioch D, Hajduch M. Clinical relevance of KRAS in human cancers. *J Biomed Biotechnol.* 2010;2010:150960.
33. Hunter ZR, Xu L, Tsakmaklis N, et al. Insights into the genomic landscape of MYD88 wild-type Waldenstrom macroglobulinemia. *Blood Adv.* 2018;2(21):2937-2946.
34. Hatjiharissi E, Ngo H, Leontovich AA, et al. Proteomic analysis of waldenstrom macroglobulinemia. *Cancer Res.* 2007;67(8):3777-3784.
35. Hunter ZR, Yang G, Xu L, Liu X, Castillo JJ, Treon SP. Genomics, Signaling, and Treatment of Waldenstrom Macroglobulinemia. *J Clin Oncol.* 2017;35(9):994-1001.
36. Herbaux C, Bertrand E, Marot G, et al. BACH2 promotes indolent clinical presentation in Waldenstrom macroglobulinemia. *Oncotarget.* 2017;8(34):57451-57459.
37. Guerrera ML, Tsakmaklis N, Xu L, et al. MYD88 mutated and wild-type Waldenstrom's Macroglobulinemia: characterization of chromosome 6q gene losses and their mutual exclusivity with mutations in CXCR4. *Haematologica.* 2018;103(9):e408-e411.
38. Kaya-Aksoy E, Cingoz A, Senbabaoglu F, et al. The pro-apoptotic Bcl-2 family member Harakiri (HRK) induces cell death in glioblastoma multiforme. *Cell Death Discov.* 2019;5:64.