

Impact of *PIK3CA* gain and *PTEN* loss on mantle cell lymphoma biology and sensitivity to targeted therapies

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Key Points

- *PIK3CA* gain and *PTEN* loss decrease the dependence of MCL cells on BCR signaling and antiapoptotic BCL2.
- *PIK3CA* gain and *PTEN* loss lead to complex metabolic rewiring and increased survival of MCL cells under hypoxia.

Besides many other mutations in known cancer driver genes, mantle cell lymphoma (MCL) is characterized by recurrent genetic alterations of important regulators of the phosphoinositol-3-kinase (PI3K) cascade including *PIK3CA* gains and *PTEN* losses. To evaluate the biological and functional consequences of these aberrations in MCL, we have introduced transgenic expression of *PIK3CA* (*PIK3CA* UP) and performed knockout/knockdown of *PTEN* gene (*PTEN* KO/KD) in 5 MCL cell lines. The modified cell lines were tested for associated phenotypes including dependence on upstream B-cell receptor (BCR) signaling (by an additional *BCR* knockout). *PIK3CA* overexpression decreased the dependence of the tested MCL on prosurvival signaling from BCR, decreased levels of oxidative phosphorylation, and increased resistance to 2-deoxy-glucose, a glycolysis inhibitor. Unchanged protein kinase B (AKT) phosphorylation status and unchanged sensitivity to a battery of PI3K inhibitors suggested that *PIK3CA* gain might affect MCL cells in AKT-independent manner. *PTEN* KO was associated with a more distinct phenotype: AKT hyperphosphorylation and overactivation, increased resistance to multiple inhibitors (most of the tested PI3K inhibitors, Bruton tyrosine kinase inhibitor ibrutinib, and BCL2 inhibitor venetoclax), increased glycolytic rates with resistance to 2-deoxy-glucose, and significantly decreased dependence on prosurvival BCR signaling. Our results suggest that the frequent aberrations of the PI3K pathway may rewire associated signaling with lower dependence on BCR signaling, better metabolic and hypoxic adaptation, and targeted therapy resistance in MCL.

Introduction

Mantle cell lymphoma (MCL) is a B-cell non-Hodgkin lymphoma characterized by a chronically relapsing clinical course.^{1,2} Besides the canonical translocation t(11;14)(q13;q32) leading to the overexpression of cyclin D1, many other recurrent molecular-cytogenetic events have been reported in patients with

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The full-text version of this article contains a data supplement.

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newly diagnosed MCL, including lesions of the phosphatidylinositol 3-kinase (PI3K)–protein kinase B (AKT)–mammalian target of rapamycin (mTOR) pathway. These include copy number gains of the P110a catalytic subunit of PI3 kinase (*PIK3CA*) and loss of phosphatase and tensin homologue (*PTEN*).³⁻⁸

The precise impact of these recurrent aberrations on the biology, survival, and drug resistance of MCL cells remain largely unexplored. Hyperphosphorylation of AKT kinase because of loss of *PTEN* has been reported in prognostically adverse MCL blastoid variant.⁵ Increased expression of *PIK3CA* was associated with MCL progression, whereas increased PI3K-AKT activity was detectable in patients who failed to respond to ibrutinib, an inhibitor of Bruton tyrosine kinase (BTK), a critical kinase involved in B-cell receptor [BCR] signaling.⁹⁻¹¹ We have recently demonstrated that *PIK3CA* copy number gains represent frequent genetic events associated with MCL relapse after failure of standard immunotherapy.¹² Because of this pathway deregulation, pharmacological targeting of the PI3K-AKT-mTOR cascade has been repeatedly investigated in MCL, either as a single agent or in combination with other targeted agents, having promising results.¹³⁻²⁰

In this study, we aimed to investigate the exact consequences of *PIK3CA* gain and *PTEN* loss on the biology of MCL cells and on the sensitivity/resistance to PI3K, BTK, AKT, and BCL2 family inhibitors.

Materials and methods

Cell lines and clones

MCL cell lines (MINO, Z138, and JEKO-1) were purchased from German Collection of Microorganisms and Cell Cultures or the American Tissue Culture Collection. UPF1H and UPF19U were derived in our laboratory from leukemized peripheral blood of patients with chemotherapy-resistant MCL. Generation of cells with transgenic *PIK3CA* (over)expression (*PIK3CA* UP) or with *PTEN* knockout (*PTEN* KO) and cultivation conditions are further described in the supplemental Material.

Cytotoxic agents

Specific PI3K inhibitors, AKT inhibitor, BTK inhibitor (BTKi), and BCL2 family inhibitors are described in the supplemental Material.

Cytogenomic analyses

Copy number alteration analyses of the *PIK3CA*, *PTEN*, and *TP53* genes were performed either by interphase fluorescence in situ hybridization (FISH) or by array-comparative genomic hybridization/single-nucleotide polymorphism, depending on the availability of material, as part of the routine diagnostic procedure. Detailed protocol description is provided in the supplemental Material.

Generation of cell lines with target genes knockout

Detailed protocol description is provided in the supplemental Material.

Generation of cell lines with target genes overexpression

Detailed protocol description is provided in the supplemental Material.

Cell lines transfection

To introduce all plasmids, we used electroporation according to the manufacturer's instructions (Neon, ThermoFisher Scientific). A total of 1.2 million cells per sample were used for the 100- μ L electroporation system. Cells were washed once by phosphate-buffered saline and resuspended in 120 μ L of electroporation R buffer together with plasmid DNA (12 μ g for single KO plasmid for green fluorescence protein (GFP) and B-cell receptor knockout (BCR KO); 5 + 5 + 5 μ g for 3 plasmids necessary for *PTEN* KO GFP KI; and 6 + 4 μ g for sleeping beauty donor and transposase plasmids, respectively, for stable overexpression) to perform electroporation. Cells were then resuspended in 3 mL of appropriate cell culture media without antibiotics for further experiments or selection. Electroporation conditions for individual cell lines were as follows (voltage, pulse length, number of pulses): Jeko-1 1500 V, 20 ms, 1 pulse; Mino 1300 V, 30 ms, 1 pulse; UPF1H 1500 V, 10 ms, 3 pulses; UPF19U 1500 V, 10 ms, 3 pulses; Z138 1600 V, 20 ms, 1 pulse.

Measurement of AKT activity in living cells

Detailed protocol description is provided in the supplemental Material.

Apoptosis and proliferation assays

Detailed protocol description is provided in the supplemental Material.

Western blotting

Detailed protocol description is provided in the supplemental Material.

Coimmunoprecipitation assay

Detailed protocol description is provided in the supplemental Material.

In vitro assays under hypoxia

The in vitro experiments under hypoxic conditions were implemented in Coy hypoxic box. Detailed protocol description is provided in the supplemental Material.

OCR and ECAR assays

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements were performed using a Seahorse XFe96 analyzer (Agilent Technologies). On the day of the experiment, cells were seeded on XFe96 cell culture microplates coated with Cell-Tak (Corning; 5×10^4 cells per well), in Seahorse base RPMI medium and incubated at 37°C without CO₂ for 1 hour. The base medium (180 μ L per well) was supplemented with 500-mM pyruvate, 2-mM L-glutamine, 10-mM glucose for OCR measurement and with 2-mM L-glutamine for ECAR measurement. The mito stress test and glycolytic stress test are further described in the supplemental Material.

Statistical analysis

To assess the statistical significance of experiments, unpaired Student *t* test or ordinary 1-way analysis of variance was used (GraphPad Prism-10). *P* value <.05 represents statistical significance. Densitometry was carried out using Image-Lab Bio-Rad-6.1 software.

Results

***PIK3CA* copy number gain and *PTEN* deletion are common aberrations in patients with MCL**

To determine the prevalence of *PIK3CA* copy number variants and *PTEN* deletions, we carried out FISH and array-comparative genomic hybridization analyses of 61 consecutive samples obtained from patients with newly diagnosed MCL. Forty-three percent of patients had gain of 1 copy of *PIK3CA* gene, 7% patients had monoallelic deletion of *PTEN*, and 1 case had both. There was no obvious correlation between *TP53* deletion and *PIK3CA* gain. Interestingly, *PTEN* deletion was found exclusively in the context of concurrent *TP53* deletion, but the relatively low number of analyzed patients precludes us from drawing any statistically significant correlations (supplemental Table 1).

***PTEN* KO but not *PIK3CA* overexpression show hyperphosphorylation and overactivation of AKT**

Overexpression of *PIK3CA* in MINO, Z138, UPF1H, UPF19U, and JEKO-1 *PIK3CA* UP cells and the loss of *PTEN* expression in UPF1H, MINO, Z138, and JEKO-1 *PTEN* KO cells were confirmed by western blotting (Figure 1A; supplemental Figure 1). UPF19U and MINO *PTEN* knockdown (*PTEN* KD) manifested a decreased expression of *PTEN* (supplemental Figure 1). Except for JEKO-1, all *PTEN* KO cells had hyperphosphorylation of the key downstream target of PI3K, the AKT kinase (Figure 1A; supplemental Figure 1). In addition, all *PTEN* KO/KD cells had increased AKT activity by fluorescence resonance energy transfer (FRET) assay (Figure 1B; supplemental Figure 2). In contrast, transgenic (over)expression of *PIK3CA* was not associated with consistent changes in AKT phosphorylation or AKT activity (Figure 1C). To determine the extent and potential correlation of *PTEN* expression and AKT phosphorylation in MCL cells, we conducted western blot profiling of 32 primary MCL samples obtained from patients with newly diagnosed and relapsed MCL. Ten of 32 patient (P) samples (P3, 6, 15, 16, 19, 20, 24, 30, 31, and 32) had higher levels of AKT phosphorylation, all accompanied by lower or no expression of *PTEN*. Three patients had undetectable *PTEN* protein (P13, 30, and 31; supplemental Figures 3 and 4A). From these, 2 patients (P13 and 30) had no genetic copy number loss of *PTEN* gene by standard FISH, suggesting alternative mechanism of loss of *PTEN* protein expression. Patients with any kind of *PTEN* genetic copy number loss (P29 and 31) had low or undetectable expression of *PTEN* protein. Of note, higher levels of AKT phosphorylation were associated with lower expression of *PTEN* protein (supplemental Figure 4B).

Loss of *PTEN* expression and gain of *PIK3CA* both decrease dependence of MCL cells on prosurvival signaling from BCR

We have clearly documented before that BCR signaling critically contributes to PI3K/AKT signaling activation in lymphomas. The degree of PI3K/AKT activation is largely dependent on BCR surface abundance.²¹ Therefore, we tested whether PI3K/AKT hyperactivation (triggered by *PTEN* KO or *PIK3CA* overexpression) can decrease the dependency of MCL cells on upstream BCR-mediated PI3K/AKT activation. We have performed BCR knockout in 3 *PTEN* KO cell line variants (MINO, UPF1H, and JEKO-1 *PTEN*/BCR KO subclones) and in 3 *PIK3CA* UP cell line

variants (MINO, UPF1H, and JEKO-1 *PIK3CA* UP/BCR KO subclones). Loss of *PTEN* partially decreased the dependence on the prosurvival signaling from the BCR (Figure 1D). Partial *PTEN* KO-mediated BCR KO rescue was detectable especially in cell lines with large increase of AKT activity after *PTEN* KO (Figure 1B) and was consistent with presumed “chronic active” type of BCR signaling in MCL that activates multiple signaling pathways than just PI3K/AKT.²² Curiously, in 2 of 3 tested *PIK3CA* UP/BCR KO subclones (MINO and JEKO-1 but not UPF1H), gain of *PIK3CA* also decreased the dependence on BCR prosurvival signaling, although to a lower extent in comparison with *PTEN*/BCR KO subclones (Figure 1E). As for the remaining MCL cell lines used in this study, Z138 cells are BCR independent (supplemental Figure 3), and UPF19U cells do not express BCR. Additional analysis in MINO and JEKO-1 cell lines showed that *PTEN* KO supported growth specifically in BCR KO cells and did not affect (JEKO-1) or slightly decreased (MINO) the growth of parental cell lines with intact BCR (supplemental Figure 6). *PI3KCA* overexpression increased the growth of MINO cells, more so in BCR-negative variant, and did not affect the cell growth of parental and BCR KO JEKO-1 cells (supplemental Figure 6). *PTEN* KD in MINO cell line did not rescue the cells from BCR KO (supplemental Figure 7).

Importantly, after BTK inhibition by ibrutinib, *PTEN* KO cells showed lower dependence on BCR-mediated AKT activation compared with parental cell lines (as detected by genetically encoded FRET-based AKT activity biosensor; Figure 1F; supplemental Figure 8), which was to a lesser extent detectable also in MINO *PTEN* KD cells (supplemental Figure 9).²³ This implies that the loss of *PTEN* overcomes ibrutinib-mediated decrease of AKT activity, which should translate into BTK inhibition resistance. Indeed, proliferation assays confirmed that *PTEN* KO cells were more resistant to ibrutinib (Figure 1G). On the contrary, *PIK3CA* overexpression did not consistently increase AKT activity and caused a paradoxical AKT activity decrease in several cell lines (Figure 1C). In accordance with this, AKT activity decrease after ibrutinib treatment was not rescued by *PIK3CA* overexpression (supplemental Figure 10), and no changes in the sensitivity to ibrutinib were detected (supplemental Figure 11).

Loss of *PTEN* and gain of *PIK3CA* affect metabolic activity and survival of MCL cells under hypoxia

The PI3K-AKT signaling pathway plays a pivotal role in the regulation of cell energy-metabolic pathways. Therefore, we used Seahorse analyzer to assess the potential impact of *PIK3CA* gain or *PTEN* loss on metabolic reprogramming of MCL. In UPF1H cells, both *PTEN* KO and *PIK3CA* overexpression decreased basal as well as maximal respiration (Figure 2A). Interestingly, these 2 genetic alterations had different effects on glycolysis in UPF1H cells. *PTEN* KO massively increased basal glycolysis (with almost no further glycolytic reserve left). Basal glycolysis was not affected by *PIK3CA* overexpression; however, *PIK3CA* overexpression decreased overall glycolytic capacity and glycolytic reserve (Figure 2B). In MINO cells, *PIK3CA* overexpression had a similar effect as in UPF1H (decreased maximal respiration and decreased glycolytic parameters); however, it was much less pronounced. *PTEN* KO did not affect respiration or glycolysis in UPF1H cells (Figure 2C-D). Substantial decrease of mitochondrial function, accompanied by increased basal glycolysis in *PTEN* KO cells,

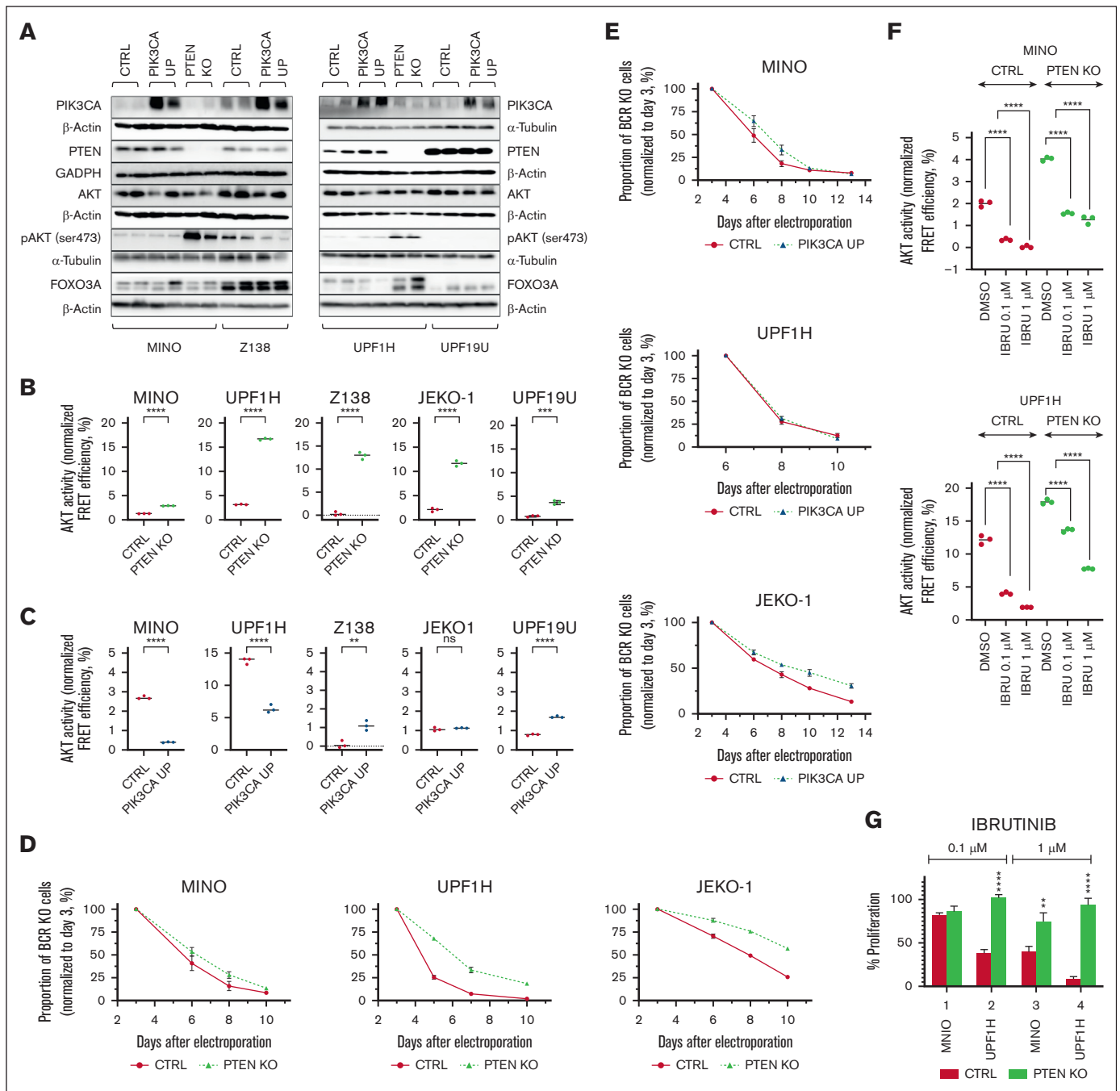


Figure 1. Impact of PTEN loss and PIK3CA gain on AKT activity and BCR prosurvival signaling. Loss of *PTEN* expression but not *PIK3CA* (over)expression is associated with hyperphosphorylation and overactivation of AKT. On the contrary, both loss of *PTEN* and, to a lesser extent, gain of *PIK3CA* decrease dependence on the prosurvival signaling from BCR. (A) Western blot. Confirmation of the upregulation of *PIK3CA* in the 4 *PIK3CA* UP cell lines MINO, Z138, UPF1H, and UPF19U and the loss of *PTEN* in the *PTEN* KO clones MINO and UPF1H. Increase of phospho-AKT (ser 473) expression in both *PTEN* KO cell lines MINO and UPF1H (for each sample, $n = 2$). (B) AKT activity as measured using genetically encoded FRET-based biosensor. Significantly increased AKT kinase activity in all *PTEN* KO cell lines compared with respective control cell lines; technical triplicates; (C) FRET assay. Variable effect of *PIK3CA* (over)expression on AKT activity; technical triplicates. (D) *PTEN* loss increases survival of MCL cells with knockout of BCR gene ($n = 3$). (E) Transgenic (over)expression of *PIK3CA* in MINO and JEKO-1 but not in UPF1H increases survival of MCL cells with knockout of BCR gene ($n = 3$). (F) AKT activity as measured using genetically encoded FRET-based biosensor. AKT activity in *PTEN* KO cell lines is higher than that of respective cell lines. In addition, after exposure to BTKi ibrutinib for 3 hours, AKT activity in *PTEN* KO cell lines remains higher than AKT activity in the respective control cell lines ($n = 3$). (G) Proliferation assay implemented 72 hours after exposure to ibrutinib (0.1 and 1 μM); the cellular proliferation of the treated cells was normalized to the cellular proliferation of the untreated cells ($n = 3$); data are represented as mean \pm standard deviation (SD); * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$; (N) represents the number of biological replicates. CTRL, controls; DMSO, dimethyl sulfoxide; ns, not significant.

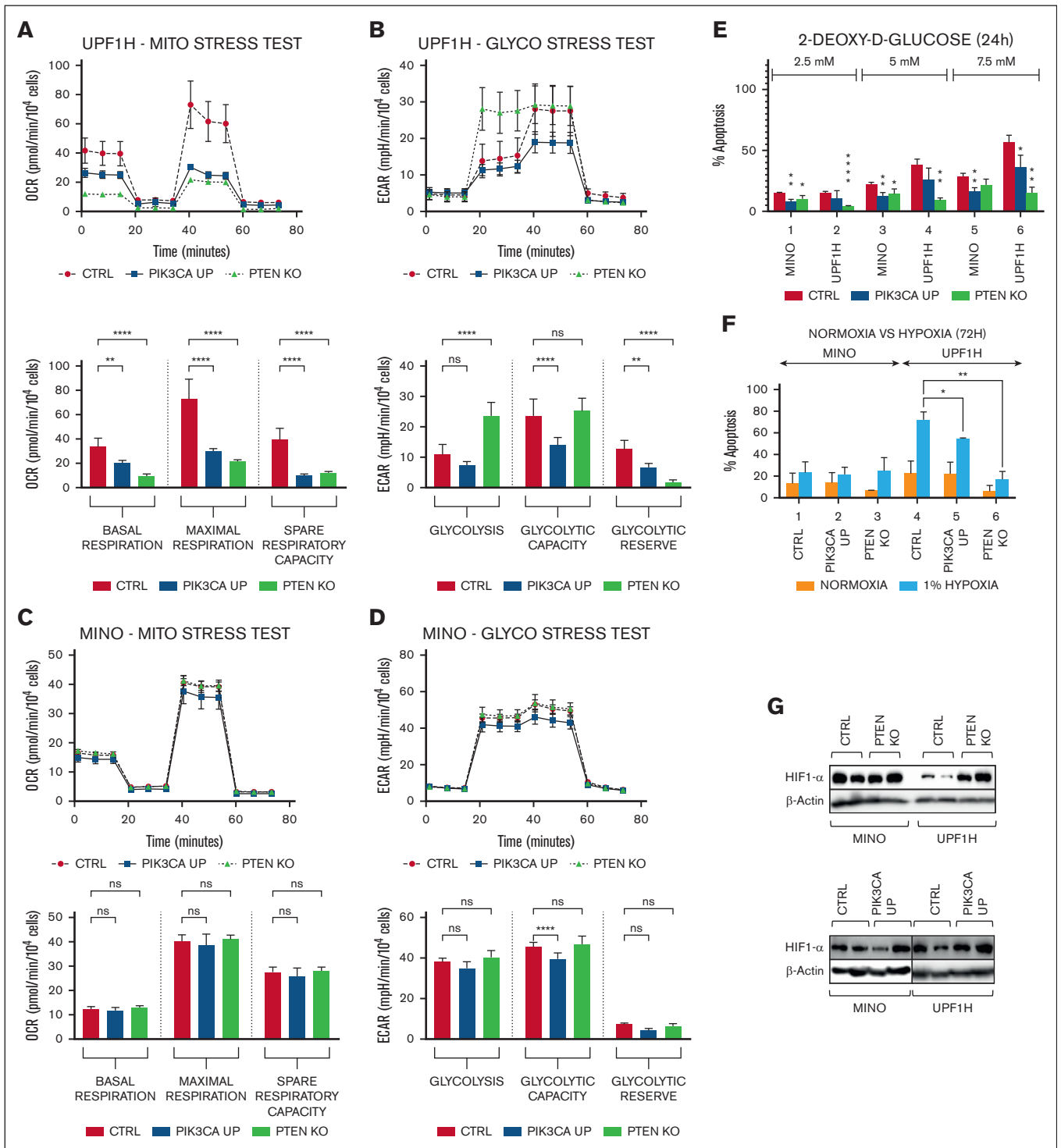


Figure 2. *PTEN* KO and *PIK3CA* UP cell lines have changed the activity of key energy-metabolic pathways and have increased survival under hypoxia. (A-D) Mitochondrial stress tests and glycolytic stress tests performed using Seahorse analyzer. (A) Decrease in the basal respiration, maximal respiration, and spare respiratory capacity in UPF1H *PIK3CA* UP and *PTEN* KO cells compared with UPF1H unmodified cell line; measured by OCR. (B) Increase in basal glycolysis in UPF1H *PTEN* KO cells, decrease of glycolytic capacity in UPF1H *PIK3CA* UP cells, and decrease of glycolytic reserve in both tested variants; measured by ECAR. (C) No significant changes of mitochondrial function in MINO *PIK3CA* UP modified cells compared with MINO CTRL. (D) Slight decrease of glycolysis parameters in MINO *PIK3CA* UP modified cells compared with MINO CTRL. (E) Number of apoptotic cells 24 hours after exposure to the inhibitor of glycolysis 2-DG (2.5, 5, and 7.5 mM); apoptosis of the treated cells was normalized to the apoptosis of the untreated cells (n = 3). (F) Increased survival of *PTEN* KO and *PIK3CA* UP cell lines after 72-hour exposure to 1% hypoxia; apoptosis of the cells cultured for 72 hours under 1% hypoxia (as well as apoptosis of the cells cultured for 72 hours in parallel under normoxia) was normalized to the apoptosis of the cells before placement into hypoxia (n = 3). (G) Western blot analysis of HIF1- α in *PTEN* KO and *PIK3CA* UP cell lines compared with respective control cell lines (for each sample, n = 2); data are represented by means \pm SD; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001; (N) represents the number of biological replicates. CTRL, XXX; ns, not significant.

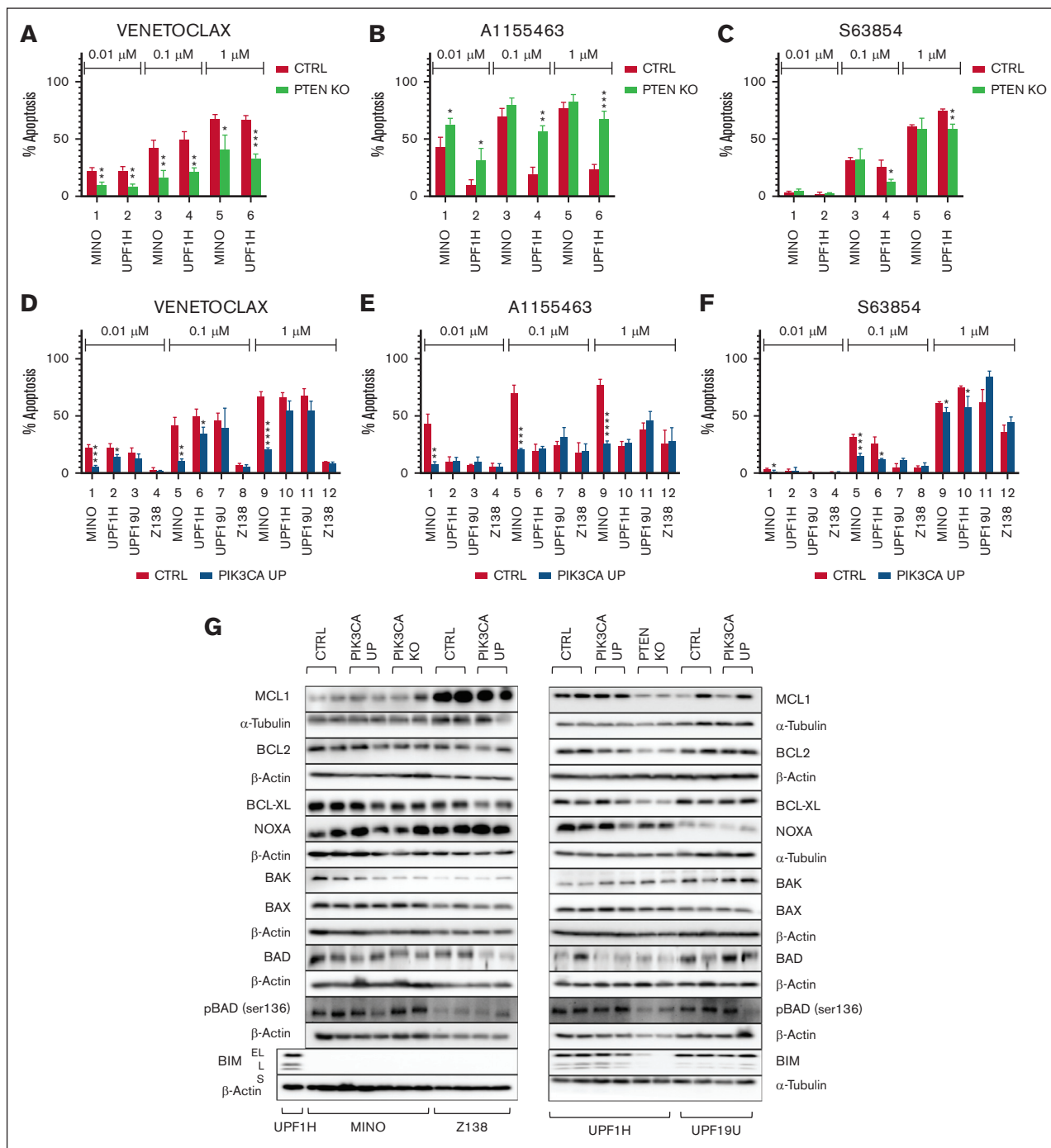


Figure 3. *PIK3CA* UP and *PTEN* KO leads to different sensitivities to BH3 mimetics. (A-F) Number of apoptotic cells 24 hours after exposure to the BH3 mimetics venetoclax (0.01, 0.1, and 1 μM), S63845 (0.01, 0.1, and 1 μM), and A1155463 (0.01, 0.1, and 1 μM). Apoptosis of the treated cells was normalized to the apoptosis of the untreated cells (n = 3). (G) Western blot analysis of selected BCL2 family proteins in *PIK3CA* UP and *PTEN* KO cell lines compared with the respective control MCL cell lines (for each sample, n = 2); data are represented by means ± SD; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001; (N) represents the number of biological replicates.

suggest increased glycolysis to oxidative phosphorylation (ox/phos) ratio in *PTEN* KO as well as *PIK3CA* UP UPF1H cells, which was not clearly detectable in MINO cells.

Functionally, both *PTEN* KO and *PIK3CA* UP UPF1H and MINO cell lines were generally more resistant to 2-deoxy-glucose (2-DG), an inhibitor of glycolysis (Figure 2E). Similar to metabolic changes,

this phenotype was more prominent in UPF1H cells. MINO *PTEN* KO did not show increased resistance to 2-DG compared with parental cell line (supplemental Figure 12).

Increased glycolytic rate is characteristic for malignant cells growing under hypoxia. Therefore, we tested the survival of modified cell lines under 3-day exposure to hypoxia (1% oxygen).

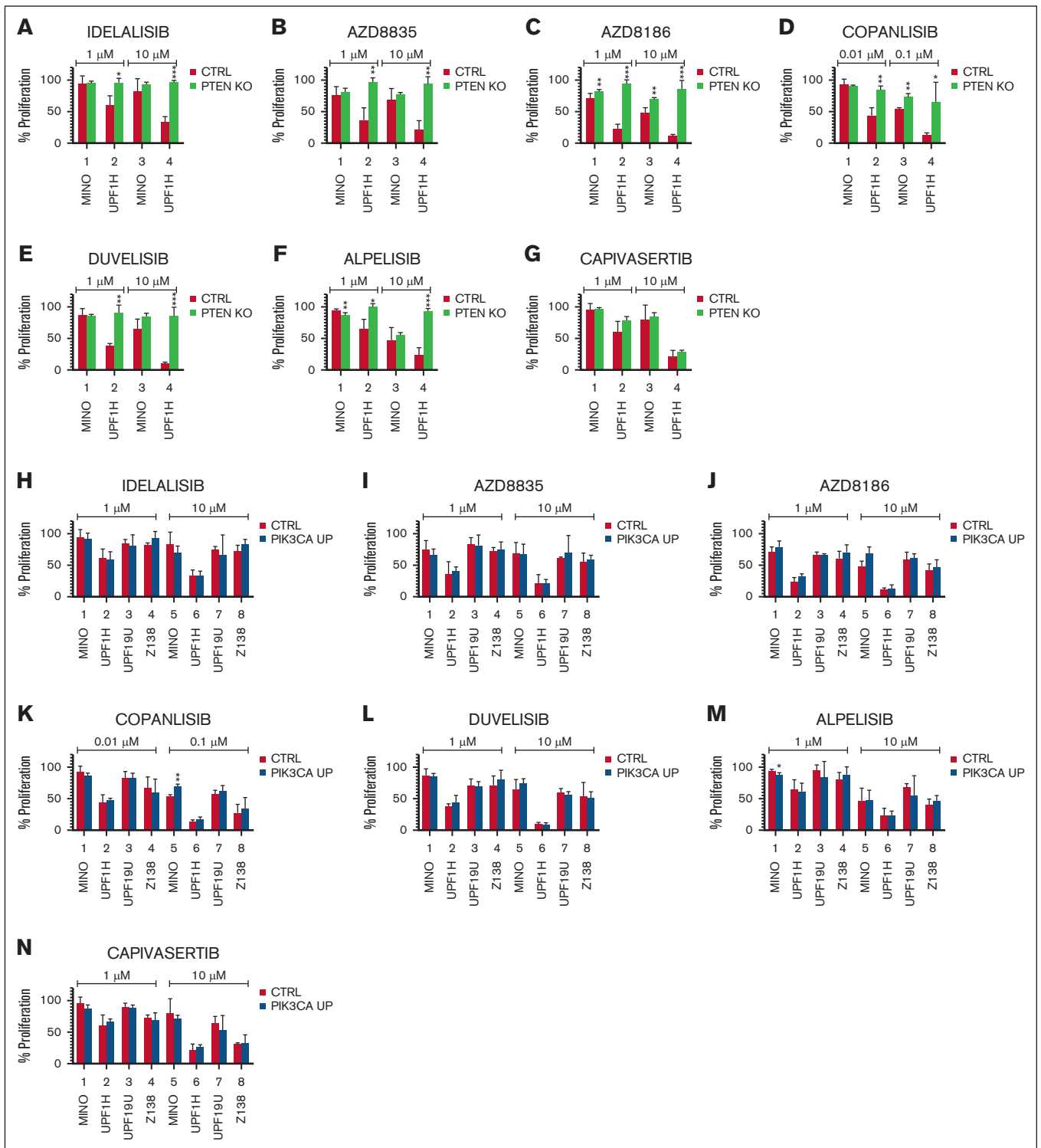


Figure 4. *PTEN* KO and, to a lesser degree, *PIK3CA* overexpression mediated resistance to a panel of *PI3K* inhibitors. (A-N) Proliferation assays implemented 72 hours after exposure to the indicated agents: idelalisib (1 and 10 μ M), duvelisib (1 and 10 μ M), AZD8186 (1 and 10 μ M), AZD8835 (1 and 10 μ M), alpelisib (1 and 10 μ M) (F), copanlisib (0.01 and 0.1 μ M), and capivasertib (1 and 10 μ M); the cellular proliferation of the treated cells was normalized to the cellular proliferation of the untreated cells (n = 3); data are represented as means \pm SD; * P < .05; ** P < .01; *** P < .001; **** P < .0001; (N) represents the number of biological replicates. CTRL, control.

UPF1H *PTEN* KO and *PIK3CA* UP cells were both more resistant to long-term exposure to 1% hypoxia (Figure 2F). UPF1H *PTEN* KO cells and, to a lesser extent, UPF1H *PIK3CA* UP cells had higher levels of hypoxia induced factor 1 (HIF1)-alpha protein by western blotting than the parental UPF1H cells (Figure 2G; supplemental Figure 13). In contrast, MINO cells were generally resistant to low tensions of oxygen, and therefore, no significant differences were observed between *PTEN* KO or *PIK3CA* UP modified cells and the parental MINO cells regarding differences in growth under hypoxia and HIF1-alpha upregulation (Figure 2F-G; supplemental Figure 13).

MCL *PIK3CA* UP and *PTEN* KO cell lines have changed sensitivity to BH3 mimetics

Given the important antiapoptotic role of the PI3K/AKT pathway and the clinical potential of BH3 mimetics, we also tested the cytotoxic efficacy of selected BH3 mimetics in the context of *PTEN* KO and *PIK3CA* overexpression. To investigate whether partial loss of *PTEN* affects sensitivity to BH3 mimetics in MCL, we tested the same panel of BCL2 family inhibitors on the MINO *PTEN* KD cell line.

Although all tested *PTEN* KO cells were more resistant to the BCL2 inhibitor venetoclax (VEN), their sensitivity to the BCL-XL inhibitor A1155463 was increased (Figure 3A-C). MINO *PTEN* KD cells were also more resistant to VEN and manifested no change in their sensitivity to A1155463 (supplemental Figure 14A-B). Two of 4 tested *PIK3CA* UP cells (UPF1H and MINO) were more resistant to VEN and S63845, a MCL1 inhibitor (Figure 3D,F).

To decipher the molecular mechanisms underlying the observed changes in the sensitivity to the tested BH3 mimetics, we implemented western blot analysis to determine the expression of key BCL2 family proteins and immunoprecipitated BCL2 and BCL-XL to identify their respective binding partners.

UPF1H *PTEN* KO cells had significantly decreased the expression of proapoptotic BCL2L11/BIM (MINO cells have biallelic deletion of BIM). MINO *PTEN* KO cells expressed more phospho-BAD (Figure 3D). Immunoprecipitation experiments demonstrated decreased amount of proapoptotic BIM protein on BCL-XL and BCL2 in UPF1H *PTEN* KO cells. On the contrary, there was a decreased BAX interaction with BCL2 in MINO *PTEN* KO cells (supplemental Figure 15).

PTEN KO and, to a lesser degree, *PIK3CA* overexpression mediated resistance to a panel of PI3K inhibitors

To explore the therapeutic consequences of *PTEN* loss and *PIK3CA* gain, we implemented a cytotoxic screen of the established *PIK3CA* UP and *PTEN* KO/KD cells using a panel of selected pathway inhibitors including PI3K inhibitors (idelalisib, duvelisib, AZD8186, AZD8835, alpelisib, and copanlisib) and an AKT inhibitor capivasertib. For the functional assays, we used 2 *PTEN* KO (UPF1H and MINO), 1 *PTEN* KD (MINO), and 4 *PIK3CA* UP cell lines variants (UPF1H, MINO, UPF19U, and Z138). Loss of *PTEN* correlated with increased resistance to PI3K inhibitors (Figure 4A-F). Although UPF1H *PTEN* KO cells were more resistant to all tested PI3K inhibitors, MINO *PTEN* KO cells

were more resistant to AZD8186 and copanlisib. MINO *PTEN* KD were more resistant to copanlisib. Both *PTEN* KO cell lines and MINO *PTEN* KD retained sensitivity to AKT inhibitor capivasertib (Figure 4G; supplemental Figure 16). With few exceptions, gain of *PIK3CA* was not associated with significant changes in the sensitivity to PI3K inhibitors or capivasertib (Figure 4H-N).

Discussion

Aberrant overactivation of the PI3K-AKT signaling pathway has been repeatedly reported in MCL and was associated with blastoid morphology and resistance to BTKis.^{5,7,9,10,24,25} In this study, we confirmed that gain of *PIK3CA* and deletion of *PTEN* are recurrent aberrations in patients with newly diagnosed MCL. Several studies demonstrated that *PTEN* is a haploinsufficient tumor suppressor gene.²⁶⁻²⁸ We also demonstrated that patients with any kind of genetic copy number loss of *PTEN* (as determined by locus specific FISH) had lower *PTEN* protein than patients with intact *PTEN* gene. In addition, low or even undetectable *PTEN* protein was observed in several MCL samples obtained from patients with intact *PTEN* gene. Our results thus confirm observations from previous studies that *PTEN* can be downregulated or completely silenced either by *PTEN* gene promoter hypermethylation or by diverse posttranscriptional and posttranslational modifications of *PTEN* messenger RNA or *PTEN* protein.²⁹⁻³² Interestingly, the most frequent type of aberration was a gain of just 1 copy of *PIK3CA* gene found in 43% of patients. We may speculate that fine-tuning of the PI3K-AKT pathway, rather than massive uncontrolled overactivation, is needed for accurate benefits from its activation in MCL cells. This is in line with previous reports by Shojaee et al who demonstrated that both the blockage and massive overactivation of PI3K-AKT is detrimental for malignant cells.³³ Importantly, FISH analysis was performed only in patients with bone marrow involvement ($\geq 20\%$ infiltration). Therefore, frequency of *PIK3CA* gains in patients with low or no bone marrow infiltration remains unknown. Frequencies of *PIK3CA* gains and *PTEN* losses at the first or subsequent disease relapses also remain elusive. However, our recent whole-exome sequencing-based study on clonal evolution of 25 patients with MCL at diagnosis and at first relapse (after failure of standard immunochemotherapy) revealed that *PIK3CA* gain is one of the most frequently identified copy number variant associated with MCL relapse.¹²

Surprisingly, transgenic (over)expression of *PIK3CA* was not associated with measurable changes in the phosphorylation or activity levels of AKT kinase or increased resistance to a wide spectrum of tested PI3K inhibitors or the BTKi ibrutinib in the majority of tested MCL cell lines. On the contrary, most of the tested MCL cell lines with *PIK3CA* overexpression were less sensitive to BH3 mimetics. Molecular mechanisms underlying the observed increase in resistance to BH3 mimetics, however, remain elusive. Importantly, *PIK3CA* gain variably decreased the dependence of the tested MCL cell lines on prosurvival signaling from the BCR, decreased the levels of ox/phos, and increased resistance to 2-DG. Generally, these changes may suggest that *PIK3CA* gain contributes to increased survival of MCL cells, especially under hypoxia. Even though precise molecular mechanisms that mediate these observations remain largely elusive, unchanged activity and phosphorylation status of AKT suggest that consequences of *PIK3CA* gain are mediated independently of AKT. This is supported by a study in endometrial carcinoma. Holst et al showed that

endometrial tumors with *PIK3CA* amplification were associated with aggressive behavior; however, had decreased levels of activated AKT at the same time.³⁴ AKT-independent cancer-promoting effect was reported also for *PIK3CA* mutations in breast cancer.³⁵

In contrast to *PIK3CA* (over)expression, knockout of *PTEN* (which mimics complete loss of PTEN protein/function) was associated with a distinct phenotype, including hyperphosphorylation and over-activation of AKT kinase, increased resistance to most of the tested PI3K inhibitors, resistance to the BTKi ibrutinib and BCL2 inhibitor VEN, markedly decreased dependence on prosurvival BCR signaling, and metabolic reprogramming of MCL cells with increased glycolytic rate and increased resistance to 2-DG. Importantly, sensitivity to AKT inhibitor capivasertib remained unchanged, which is consistent with data published thus far.³⁶ Interestingly, sensitivity to BCL-XL inhibitor A1155463 was increased. The observations from the western blot analyses (ie, downregulation of BCL2 and BIM proteins in UPF1H *PTEN* KO and downregulation of BAK1 in MINO *PTEN* KO) and immunoprecipitation experiments (ie, decreased interaction of BIM with BCL2 in UPF1H *PTEN* KO and decreased amount of BAX on BCL2 in MINO *PTEN* KO) may at least partially explain increased resistance of *PTEN* KO cell lines to VEN. However, molecular mechanisms underlying the observed increased sensitivity to BCL-XL inhibition remain elusive.

Of note, knockdown of PTEN (which mimics partial loss of PTEN protein/function) was associated with slight but significant increase in AKT activity, lower AKT activity reduction in response to ibrutinib, and increased resistance to copanlisib, ibrutinib, VEN, and S63854. This is important because our data showed that even partial loss of PTEN protein negatively correlated with sensitivity to BTKis. PTEN might thus represent an important predictive factor of sensitivity to BTKi. However, this correlation must be confirmed on primary MCL samples before and after failure of BTKi therapy.

We proved that both aberrations (ie, gain of *PIK3CA* and loss of *PTEN*) might lead to metabolic reprogramming of MCL cells, which results in significant changes in the rates of glycolysis and ox/phos, increased resistance to 2-DG, and increased survival under hypoxia. Previous studies demonstrated that hypoxia adaptation was associated with allelic loss or inactivation of *PTEN*.^{37,38} Conversely, loss of *PTEN* facilitated HIF1-mediated gene expression.³⁹⁻⁴¹ Metabolic reprogramming belongs to hallmarks of cancer.⁴²⁻⁴⁴ Overactivation of PI3K pathway promotes glycolysis in an AKT-dependent and -independent manner. One example of AKT-independent activation of glycolysis is the effect of the above-mentioned *PIK3CA* mutation.^{45,46} Together with the observed decreased dependence on the prosurvival signaling from BCR, we propose that both *PTEN* loss and *PIK3CA* gain foster survival of MCL cells under hypoxia.

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Conclusion

Our results suggest that frequent aberrations of the PI3K pathway observed in MCL may rewire associated signaling with lower dependence on BCR signaling, better metabolic and hypoxic adaptation, and targeted therapy resistance.

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Authorship

Contribution: P.K. and O.H. designed the research, analyzed data, and supervised the preparation of the manuscript; N.B. performed most of the research and wrote the manuscript; J.S., K.K., A.R., and V.H. derived genetically modified cell lines, performed measurements of AKT activity, and performed experiments with the BCR KO; L.A. and D.S. implemented Seahorse analyses; K.S., A.B., and Z.Z. performed FISH analyses; L.D. and A.D. performed hypoxic experiments; and M.T. and R.E.D. analyzed data and reviewed the manuscript.

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