**Phosphatidylinositol 3'-Kinase Catalytic Subunit α Gene Amplification Contributes to the Pathogenesis of Mantle Cell Lymphoma**

Amanda Psyrri,1,6 Sotirios Papageorgiou,1 Elisavet Liakata,1 Andreas Scorilas,2 Dimitra Rontogianni,3 Christos K. Kontos,2 Pinelopi Argyriou,3 Dimitrios Pectasides,1 Nikolaos Harhalakis,4 Vassiliki Pappa,1 Aggeliki Kolialexi,5 Christina Economopoulou,1 Frida Kontsioti,1 Eirini Maratou,1 George Dimitriadis,1 Panagiota Economopoulou,1 and Theofanis Economopoulos1

**Abstract**

**Purpose:** Activation of phosphatidylinositol 3'-kinase pathway is implicated in the pathogenesis of mantle cell lymphoma (MCL). The genetic change in *phosphatidylinositol 3'-kinase catalytic subunit α* (**PIK3CA**) in MCL has not been identified.

**Experimental Design:** Thirty-five primary MCL cases and 2 MCL cell lines (GRANTA-519 and Rec-1) were used to investigate somatic mutation and gene copy number of **PIK3CA**. Gene copy number was determined using quantitative real-time PCR and fluorescence in situ hybridization. We used quantitative real-time reverse transcription-PCR to measure **PIK3CA** transcription levels. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and phosphorylated AKT protein levels were analyzed using Western blotting and immunohistochemistry. Flow cytometry was used to assess apoptosis after treatment of MCL cell lines and one control cell line with LY294002, a specific inhibitor of PI3KCA.

**Results:** Fifteen of 22 (68%) MCL cases and the MCL cell lines harbored a gain (≥3) of **PIK3CA** gene copy number. In addition, cases with increased **PIK3CA** gene copy number had elevated **PIK3CA** mRNA levels. Furthermore, amplification of **PIK3CA** correlated with the status of AKT phosphorylation in 7 of 12 (58%) primary MCL cases. Inhibition of **PIK3CA** induced increased apoptosis in the MCL cell lines. PTEN protein expression was present in all 14 primary MCL cases and cell lines by Western blotting, whereas 5 of 33 (15%) cases tested by immunohistochemistry had loss of PTEN expression.

**Conclusions:** We conclude that a gain of gene copy number of **PIK3CA** is frequent genetic alteration that contributes to MCL progression. **PIK3CA** is a promising therapeutic target in MCL. (Clin Cancer Res 2009;15(18):5724–32)

Mantle cell lymphoma (MCL) is now recognized as a distinct clinicopathologic subtype of B-cell non–Hodgkin’s lymphoma with well-characterized morphology, a distinct immunophenotypic profile, specific karyotypic abnormality, and even a unique underlying molecular alteration (1). MCL comprises approximately 3% to 10% of non–Hodgkin’s lymphoma and is characterized by an aggressive clinical course and poor prognosis with a median survival of 3 to 5 years (2, 3). Although front line therapy induces a high rate of complete remission, relapse is inevitable and new treatments are needed for relapsed MCL (4).

The genetic hallmark of MCL is the chromosomal translocation (t(11;14)(q13;q32)), which results in deregulated aberrant expression of cyclin D1 (5–7). However, the concept that MCL represents a distinct clinicopathologic and molecularly defined entity does not preclude the existence of additional distinctive disease subsets that could define disease heterogeneity. The genetic change in **PIK3CA** is associated with disease subsets that could define disease heterogeneity. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org). Portions of this material were presented at the 43rd Annual Meeting of the American Society of Clinical Oncology, June 2007. A. Psyrri and S. Papageorgiou contributed equally to this work.

**Requests for reprints:** Amanda Psyrri, Rimini 1, Second Department of Internal Medicine, Propaedeutic, University of Athens, University General Hospital “Attikon,” Haidari, Athens, Greece. E-mail: diamando.psyrri@yale.edu.

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PIK3CA Gene Amplification in MCL

Deregulated signaling through phosphatidylinositol 3'-kinase (PI3K) pathway is common in many types of malignancies, including mantle cell lymphoma. Dissecting the molecular events associated with activation of this pathway in mantle cell lymphoma presents an important challenge that has implications for the development and clinical testing of PI3K pathway inhibitors. We show that PI3K catalytic subunit α (PIK3CA) gene amplification is highly correlated with activation of the main PI3K effector AKT in vivo. Loss of the tumor suppressor protein phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was found to be another mechanism of AKT activation. In addition, PIK3CA gene amplification and PTEN inactivation were found to be mutually exclusive, which implies that abrogation of either PIK3CA or PTEN is sufficient to activate PI3K/AKT pathway. Based on our results, determination of PIK3CA gene and PTEN protein status may have value as biomarkers for patient selection in clinical trials testing PI3K or AKT inhibitors, but this remains a conjecture. Validation of our results in a larger sample size will determine the utility of PIK3CA gene and PTEN protein status as predictive biomarkers.

Materials and Methods

Primary cases and cell lines. After institutional review board approval, 35 primary MCL cases (14 frozen and 21 paraffin-embedded specimens) were selected from the files of the Laboratory of Pathology, "Evangelismos" Hospital of Athens. All cases were reviewed by two expert hematopathologists (D.R. and P.A.) and classified according to the WHO as either typical MCL or blastic variant (29). All cases were positive for cyclin D1 and/or t(11;14) (by immunohistochemistry and/or cytogenetics) and had a minimum of 75% tumor cell infiltration.

Two MCL cell lines (Rec-1 and GRANTA-519; refs. 30, 31) and four non-MCL cell lines (Raji, SU-DHL-1, OVCAR-3, and U-937) were included in this study. Rec-1, SU-DHL-1, Raji, OVCAR-3, and U-937 were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. GRANTA-519 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at the same conditions.

DNA extraction. Genomic DNA was extracted from frozen tissue or cell pellets by using the total RNA/DNA purification kit (Macherey-Nagel) according to the manufacturer's protocol. DNA was extracted from formalin-fixed, paraffin-embedded tissue as previously described (32). Briefly, 12 sections (thickness, 10 μm) in each case were put on slides and deparaffinized in xylene (three for 5 min). The extraction was carried out with QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions.

PIK3CA mutation analysis. The primers used for PCR and sequencing were as follows: exon 9, 5'-CATTGGTGCCTCTCGTTG-3' (forward) and 5'-TGGTGACATCCGCAAATTTC-3' (reverse); exon 20, 5'-TGGGCTGAAGGAAATTGAAACG-3' (forward) and 5'-CCTATGGCAGTCCTTTGG-3' (reverse). After incubation at 95°C for 5 min, 35 cycles of amplification were done at 95°C, 56°C (exon 9) or 48°C (exon 20), and 72°C each for 1 min. An elongation step was finally followed at 72°C for 8 min. The PCR products were subjected to direct sequencing PCR of both strands with BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems). The samples were finally analyzed on an ABI 3730 genetic analyzer (Applied Biosystems).

Copy number analysis of PIK3CA with quantitative real-time PCR. PIK3CA gene copy number quantification was evaluated using quantitative real-time PCR (qRT-PCR) using the ABI 7500 Sequence Detection System instrument and software (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Entrez Gene ID: 2597) localized at 12p13 was used as a reference gene. Based on the copy number analysis, PIK3CA gene copy number quantification was evaluated using quantitative real-time PCR (qRT-PCR) using the ABI 7500 Sequence Detection System instrument and software (Applied Biosystems).
on the information of the PIK3CA and GAPDH DNA sequences, two pairs of gene-specific primers were designed. Forward and reverse primers were 5′-AGATAACTGAGAAAATGAAAGCTCACTCT-3′ and 5′-TGTTCATGGATTGTGCAATTCC-3′ (for PIK3CA) and 5′-CCTCCCGCTTCGCTCTCT-3′ and 5′-CCTGGCGACGCAAAAAGA-3′ (for GAPDH), with PCR product lengths of 104 and 65 bp, respectively. Primer concentrations were selected based on preruns using the optimization protocols from the ABI Power SYBR Green PCR Master Mix manual (Applied Biosystems). Each 10 μL real-time PCR volume contained 20 ng of extracted DNA, 1× Power SYBR Green PCR Master Mix (Applied Biosystems), and 50 μmol/L of the forward and reverse primers. For Ct determination, three parallels were assayed for each gene. The thermal cycling consisted of an initial denaturing step at 95°C for 10 min followed by 40 cycles consisting of 95°C for 15 s and 60°C for 1 min. A validation experiment, as described in ABI PRISM 7700 Sequence Detection System User Bulletin 2 (Applied Biosystems), showed equal efficiencies of target and reference amplification over a range of DNA concentrations (Fig. 1). Thus, PIK3CA gene copy number calculation could be carried out using the comparative Ct (2-ΔΔCt) method. Twenty-two MCL samples had sufficiently amplifiable GAPDH by qRT-PCR and were analyzed for PIK3CA gene status. We did not observe any deletion of GAPDH in the MCL samples. In addition to a no-template background control, DNA extracted from a specimen of normal tissue was included in the assay as a calibrator sample. As a negative control, we also used the U-937 cells, which possess two copies of PIK3CA gene and two copies of GAPDH gene, whereas the cell line OVCAR-3 was used as a positive control, as it contains six gene copies of PIK3CA gene and two copies of GAPDH gene (21). The U-937 histiocytic lymphoma cell line was used as a calibrator in order that PCRs from distinct runs become comparable. Thus, normalized results were expressed as the ratio of PIK3CA gene copies to GAPDH gene copies calculated for each MCL sample or cell line, in relation to the same ratio calculated for U-937 cells. Each real-time PCR was done in triplicate to evaluate reproducibility of data. For statistical evaluation, the cutoff line for PIK3CA was set at 3 gene copies and the cases were subdivided into two groups with respect to the PIK3CA gene amplification values: lower than 3 (no amplification) and 3 or higher (amplification).

**Fluorescence in situ hybridization.** Dual-color fluorescence in situ hybridization was done using the bacterial artificial chromosome clones RP11-466H15 and RP11-1115H8 for PIK3CA labeled with Spectrum.

![Fig. 1. Validation of the 2-ΔΔCt method. Amplification plots of PIK3CA (A) and GAPDH (B) genes. Serial dilutions of DNA were amplified by qRT-PCR using the ABI 7500 Real-Time PCR System. The software constructs amplification plots, where ΔRn is plotted versus cycle number. C, standard curves for GAPDH (black line) and PIK3CA (yellow line) amplicons. All data were fit using least-squares linear regression analysis. Both regression lines show a very good linearity (R² = 0.999 and R² = 0.993, respectively) and quite equal slopes, which implies the same amplification efficiency for both amplicons. D, ΔCt (Ct, PIK3CA - Ct, GAPDH) was calculated for each DNA dilution.](https://www.aacrjournals.org/doi/abs/10.1158/1078-0432.CCR-08-3215)
Green (kindly provided from Susanne Gollin, University of Pittsburgh, Pittsburgh, PA) and chromosome 3-satellite labeled directly in Spectrum Orange (Vysis) in accordance with the manufacturer’s instructions. The combination of two bacterial artificial chromosomes for PIK3CA ensures that the signal represents more specifically PIK3CA gene locus compared with a single bacterial artificial chromosome clone RP11-466H15. Slides were fixed using methanol/acetic acid (3:1) pretreated with RNase A and pepsin. After hybridization, slides were counterstained with 4′,6-diamidino-2-phenylindole (Sigma), mounted with antifade, and examined under a fluorescent microscope equipped with a charge-coupled device camera (Axioskop, Zeiss). From each case, 200 nuclei were analyzed and all signals were counted.

**RNA preparation and real-time RT-PCR.** Total RNA was extracted from cell lines or snap-frozen tissue using Trizol reagent (Invitrogen, Inc.) following the manufacturer’s instructions. The concentration and purity of RNA were assessed spectrophotometrically at 260 and 280 nm, and an A260/A280 ratio of 1.8±2.2 was found. Total RNA (2 μg) was reverse transcribed into first-strand cDNA using the Super-Script First-Strand Synthesis System for RT-PCR (Invitrogen) and an oligo(dT)12-18 primer, according to the manufacturer’s instructions. The final reaction volume was 20 μL.

Quantitative real-time reverse transcription-PCR was done in the ABI 7500 Real-Time PCR System using the SYBR Green chemistry. Having taken into consideration the information of the PIK3CA and GAPDH cDNA sequences, two pairs of gene-specific primers were designed. Each 10 μL real-time PCR volume contained 20 ng of cDNA, 1× Power SYBR Green PCR Master Mix, and 50 μmol/L of the forward and reverse primers. The PIK3CA real-time PCR primers were 5′-CCCAGAAGCCAGAATTAGGC-3′ and 5′-TTGAGCTGTTCTTTGTCATTTTCC-3′, producing a 94-bp PCR amplicon, and the GAPDH real-time PCR primers were 5′-CTCCTCCTCAGACTCCATTGC-3′ and 5′-CCGTTGACTCCGACCCCTAC-3′, resulting in a 116-bp PCR amplicon. The cycling conditions were as follows: a denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, and a final step for the creation of a dissociation curve, so as to distinguish the main PCR product from primer-dimers.

Equal efficiencies of target and reference amplification over a range of cDNA concentrations were shown through a validation experiment as described in ABI PRISM 7700 Sequence Detection System User Bulletin 2, and calculations were made using the comparative Ct (2¬ΔΔCt) method. GAPDH was used as an endogenous control gene for normalization of the PCR for the amount of RNA added to the

**Fig. 2.** Amplification plots of PIK3CA (left curve in each plot) and GAPDH (right curve in each plot) genes. PIK3CA gene duplication was detected by qRT-PCR using the comparative Ct method. ΔΔCt ratio (2¬ΔΔCt) defines the PIK3CA gene copy number in a healthy control sample, in lymphoma cell lines (GRANTA-519 and Rec-1), and in MCL samples. All samples were run in triplicate. A, control sample (ΔΔCt ratio = 1.00). B, gene duplication in GRANTA-519 cells (ΔΔCt ratio = 1.51). C, gene amplification in Rec-1 cells (ΔΔCt ratio = 2.35). D, gene amplification in a patient with MCL (ΔΔCt ratio = 4.18). Y axis, ΔRn; X axis, cycle number. The threshold was automatically set above the background level calculated from the baseline cycles.
reverse transcription reactions, whereas the U-937 histiocytic lymphoma cell line was used as a calibrator in order that PCRs from distinct runs become comparable. Thus, normalized results were expressed as the ratio of PIK3CA mRNA copies to GAPDH mRNA copies calculated for each MCL sample or cell line, in relation to the same ratio calculated for U-937 cells. Each real-time reverse transcription PCR was done in triplicate to evaluate reproducibility of data.

**Protein extraction and Western blot analysis.** Protein extraction from frozen tissue or cultured cells was done using the RIPA/Protein kit (Macherey-Nagel) according to the manufacturer's protocol. Quantification of the secondary antibody was done after the addition of trichloroacetic acid to the samples. On trichloroacetic acid addition, protein precipitates and causes turbidity. The degree of turbidity is used for quantification relative to a sample with known protein concentration. Protein extract (30 μg) from each sample was electrophoresed through 10% SDS polyacrylamide gel and transferred to nitrocellulose membrane (0.45 μm; Bio-Rad). Nonspecific binding sites were blocked by incubation with 5% bovine serum albumin and 5% nonfat dry milk in TBS-Tween 20 (TBST) for 1 h at room temperature. Subsequently, membranes were incubated with primary antibody to phosphorylated AKT (pAKT; Ser473; 1:1,000; Cell Signaling Technology), AKT (1:1,000; Cell Signaling Technology), and PTEN (1:250; Santa Cruz Biotechnology) overnight at 4°C. After incubation for 1 h with horseradish peroxidase–conjugated secondary antibody, anti-rabbit or anti-goat IgG (Upstate and Chemicon, respectively), immunoreactivity was visualized with SuperSignal West Pico Chemiluminescent kit (Pierce) and exposed to Kodak film for a minute.

**Immunohistochemistry.** Paraffin sections were cut at 4 μm, mounted on positively charged slides, and air dried overnight at 37°C. Before staining, the sections were deparaffinized and rehydrated using xylene, serial dilutions of ethanol (100%, 95%, 80%, and 70%), and distilled water. The slides were washed twice for 15 min with TBST (DAKO). Antigen retrieval was completed by treating the slides in antigen retrieval solution (DAKO) in a steamer for 40 min. The slides were then cooled down at room temperature for 10 min and washed twice for 5 min with TBST buffer. Then, the slides were placed in 3% hydrogen peroxide/PBS (pH 7.4; DAKO) for 10 min in the dark. The slides were washed under tap water to completely remove hydrogen peroxide, and then they were washed twice for 5 min with TBST buffer. The slides were incubated in PBS for 15 min at room temperature in a humidified chamber and then incubated with the rabbit polyclonal anti-pAKT (Ser473) antibody (1:50 dilution; Cell Signaling Technology) or with the rabbit polyclonal anti-PTEN (1:25 dilution; Zymed Laboratories) in a humidified chamber at room temperature for 60 min. After washing twice with TBST buffer for 10 min each, the slides were incubated with the secondary antibody, labeled streptavidin biotin blocking system (LSAB; DAKO), at room temperature in a humidified chamber for 20 min, and then they were washed twice with TBST buffer for 10 min each. The slides were incubated with LSAB at room temperature in a humidified chamber for 20 min, washed twice with TBST buffer for 10 min each, incubated with 3,3′-diaminobenzidine solution for 1 min at room temperature, rinsed with water to remove residue 3,3′-diaminobenzidine, counterstained with Mayer's hematoxylin (DAKO) for 3 min, and dehydrated using serial dilutions of ethanol (70%, 80%, 95%, and 100%) and xylene. As positive controls, prostatic tissue containing carcinoma and normal parenchyma (PTEN) and breast cancer biopsies (pAKT) were used.

**Immunohistochemical scoring.** According to scoring system that has been reported previously in the literature (33, 34), our pAKT or PTEN staining scoring was done as follows: stained sections were semiquantitated using light microscope at high-power field and blindly evaluated by two pathologists (D.R. and P.A.) without prior knowledge of the clinicopathologic parameters. The cells showing cytoplasm and/or nucleus staining were judged as positive. Five high-power fields in light microscope were selected randomly. The average percentage of cells staining positively was calculated in each field among 200 cells counted.

The average percentage of positive cell is designated as 0 when no tumor cell stained, 1 when 10% to 20% of cells stained, 2 when 20% to 50% of cells stained, and 3 when >50% of cells stained. The intensity of cell staining positively was categorized as follows: 0, no appreciable staining in cells; 1, barely detectable staining compared with stromal elements; 2, readily appreciable brown staining distinctly marking cell cytoplasm and/or nucleus; and 3, dark brown staining in cells completely obscuring cytoplasm and/or nucleus. Scoring was done according to product of staining intensity and average percentage of cell staining positively ranging from 0 to 6. For purposes of statistical analysis, all cases scoring product 2 or less were grouped as negative expression and all cases scoring product 3 or greater were grouped as positive expression.

**Apoptosis detection.** Two MCL cell lines (Rec-1 and GRANTA-519) and the Raji Burkitt's lymphoma cell line were treated with LY294002, a specific inhibitor of PIK3CA, and cells were harvested 24 and 48 h after incubation. The concentrations of LY294002 used were 25, 50, and 100 μmol/L. Apoptotic and dead cells were quantified by Annexin V–FITC and propidium iodide (PI) double staining using a staining kit from BD Biosciences according to the manufacturer's instructions and analyzed by flow cytometry. Briefly, cells were harvested, washed twice with cold PBS, and then resuspended in 1× binding buffer at 4°C.

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PIK3CA Gene Amplification in MCL

Results

Gain of copy number of PIK3CA gene in MCL

One mechanism of oncogenic activation of PI3K involves gene amplification (17, 21). To determine the PIK3CA gene copy number, we did qRT-PCR on 2 MCL cell lines and 35 MCL samples. Of the 35 MCL primary cases, 7 were blastoid and 28 were of classic type. Setting a cutoff line at three PIK3CA gene copies, 15 of 22 (68%) MCL samples with amplifiable GAPDH displayed PIK3CA gene amplification. Four of five analyzable blastoid cases harbored a gain of PIK3CA gene copy number. Four cases (one blastoid and three classic) showed a significant increase of gene copy number (more than 7 copies), with the highest number being 8.4. In addition, PIK3CA gene copy number was also determined in two MCL cell lines, GRANTA-519 and Rec-1, and found to be amplified (3 and 4.7 copies, respectively; Fig. 2). We also confirmed the gene copy number results of the two MCL cell lines with fluorescence in situ hybridization analysis. GRANTA-519 contained 4 to 6 (mean, 4.3) and Rec-1 contained 5 to 10 (mean, 6.2) copies per cell. Representative images are shown in Fig. 3. Thus, our data indicate that gene amplification/gain of copy number of PIK3CA is a frequent genetic alteration in MCL.

Expression of PIK3CA in MCL correlates with gene copy numbers determined by real-time PCR

To assess the relationship between amplification and expression of PIK3CA, we used qRT-PCR to measure PIK3CA transcription levels in 13 MCL cases and the 2 MCL cell lines (GRANTA-519 and Rec-1). As shown in Fig. 4, MCL cases with increased PIK3CA gene copy number (≥3) tended to have elevated mRNA expression. Thus, the increase in transcription level in MCL cases likely results from the PIK3CA gene copy number increase.

Absence of activating PIK3CA mutations in primary MCL cases and cell lines

Mutations of PIK3CA gene can cause constitutive activation of PI3K and the consequent activation of AKT pathway (35). We therefore analyzed all MCL cases and the two MCL cell lines for somatic mutations within the helical and kinase domains (exons 9 and 20) of PIK3CA gene. Sequence analysis was done to assess the two hotspot regions (exons 9 and 20), where >80% of the reported mutations are found (23). However, none of the MCL cases or cell lines were found to have activating somatic mutations (data not shown).

Biological effect of PIK3CA gene amplification in MCL

Expression of AKT, pAKT, and PTEN proteins in MCL. To determine the activation status of the PI3K/AKT pathway in MCL, we studied protein expression levels of AKT, pAKT, and PTEN in 14 frozen MCL samples and in 2 MCL cell lines (Rec-1 and GRANTA-519) using Western blot analysis. Western blot analysis revealed expression of AKT and PTEN in the 2 MCL cell lines and all 14 frozen MCL samples tested. High levels of pAKT were shown in the 2 MCL cell lines and in 5 of 14 (36%) MCL cases. Representative data from cell lines and MCL samples are shown in Fig. 5B. Three of five pAKT-positive samples were associated with high (≥3) PIK3CA gene copy number and two of them had PIK3CA gene copy number higher than 7. We also

![Fig. 4. Relative PIK3CA mRNA expression in MCL samples. MCL patients with high PIK3CA gene copy number (≥3; right column) show a quite 2-fold increase of PIK3CA mRNA expression (2.50 ± 0.57 c/c) in comparison with those with no PIK3CA gene amplification or low (<3) gene copy number (1.27 ± 0.38 c/c (left column)). c/c, PIK3CA mRNA copies/GAPDH mRNA copies (P = 0.029, Mann-Whitney nonparametric test).](image1)

![Fig. 5. Representative images from immunohistochemistry of AKT, pAKT, and PTEN proteins in MCL. A, immunohistochemistry of AKT, pAKT, and PTEN. pAKT expression nuclear and cytoplasmic (i) and predominantly nuclear (ii). PTEN-positive expression predominantly cytoplasmic (iii) and nuclear (iv). Original magnification, ×200 (i) and ×400 (ii-iv). B, Western blot analysis for AKT, activated pAKT (Ser473), and PTEN. Representative data from two MCL cell lines GRANTA-519 (G) and Rec-1 (R), a non-MCL cell line (Raji), and MCL cases. High levels of AKT were detected in all examined samples. pAKT expression was detected in samples 5, 6, and 8 and in the two MCL cell lines but not in the Raji cell line. PTEN expression was detected in all examined samples. SUDHL-1 (S) served as positive control. Actin is shown as loading control.](image2)
examined the expression status of pAKT and PTEN using immunohistochemistry in the above samples and in additional 21 paraffin-embedded specimens from MCL patients. In the immunohistochemical analysis of pAKT (Ser473) expression, 12 of 33 (36%) MCL samples showed high levels of pAKT expression (Fig. 5A, i and ii). Four of seven blastoid MCL cases analyzed were positive for pAKT. Areas with AKT phosphorylation displayed particularly prominent proliferative activity (Ki-67 labeling; data not shown). Twenty-eight of 33 (85%) specimens had positive PTEN staining, whereas 5 (15%) had no PTEN staining (Fig. 5A, iii and iv). The latter five cases with negative PTEN staining (one blastoid and four classic type) had positive pAKT staining. Seven of 12 samples (58%) with positive pAKT staining displayed high (≥3) PIK3CA gene copy number and 3 of them had gene copy number higher that 7. One sample with positive pAKT staining was associated with both loss of PTEN expression and PIK3CA gene amplification. Therefore, it seems that loss of PTEN expression and PIK3CA gene amplification are nearly mutually exclusive genetic mechanisms of AKT activation in MCL. One sample with high pAKT expression had neither PIK3CA gene amplification nor loss of PTEN expression. The results are summarized in Supplementary Table S1.

Inhibition of PIK3CA induces apoptosis in MCL cell lines
To investigate whether cell death after PIK3CA inhibition was via apoptosis, Annexin V binding and PI permeability were
examined by using flow cytometry. Nonpermeabilized cells were stained with Annexin V antibody and PI 24 and 48 hours after LY294002 treatment and analyzed by using flow cytometry (Fig. 6). In this analysis, early apoptotic cells bind Annexin V only and not PI and are visualized in the bottom-right quadrant of a two-dimensional plot. As shown in Fig. 6B, treatment with 25 μmol/L LY294002 increased the population of Annexin V–positive cells from 3% to 20% in GRANTA-519 MCL cells and from 7.3% to 20% in Rec-1 MCL cells, whereas it had no such effect in Raji control cell line.

**Discussion**

In this study, we aimed to determine the contribution of the PIK3CA gene alterations in the pathogenesis of MCL. First, we analyzed 2 MCL cell lines and 22 primary MCL cases for gain in PIK3CA gene copy number and found that PIK3CA gene amplification is a frequent event found in 68% of the cases and 2 of 2 MCL cell lines. Subsequently, we did mutational analysis in the same cohort of MCL cases and in the two MCL cell lines and found the absence of activating somatic mutations within the helical and kinase domains (exons 9 and 20) of PIK3CA gene. To investigate the functional implications of PIK3CA gain, we evaluated immunohistochemically the expression of active PIK3CA helical and kinase domains (exons 9 and 20) of PIK3CA gene. PTEN expression harbored gain of PIK3CA protein expression. Only one of the five MCL cases with loss of PTEN expression had an associated PIK3CA gene amplification in five cases that were not associated with pAKT expression. Six of 31 MCL cases (19%) and 3 of 4 cell lines had loss of PTEN expression. In concordance with our findings, PIK3CA gene mutations were not identified.

In the present study, we also found PIK3CA gene amplification in five cases that were not associated with pAKT expression. Two of them had low mRNA levels, one had very high mRNA levels, and the other two were not analyzed for PIK3CA mRNA expression levels due to lack of frozen material. There are several potential explanations for this observation. First, PIK3CA gene amplification might not directly correlate with PIK3CA protein levels due to posttranscriptional or posttranslational mechanisms, which finely tune the expression level of the catalytic subunit of PI3K. Our results, in line with those obtained by Pedrero et al. (41), suggest that epigenetic events also influence p110α expression in MCL. This lack of correlation between gene copy number and protein levels has been reported for other genes, such as EGFR and HER-2/neu (42). Another potential explanation is that the PI3K is able to promote cell survival via AKT-independent mechanisms, such as those involving the serum and glucocorticoid kinase and the cytokine-independent survival kinase (43, 44). Therefore, our results hint to multiple downstream PI3K effectors, which cross-talk in MCL pathogenesis.

In addition to PIK3CA genetic alterations and PTEN abrogation, AKT activation can occur through different mechanisms (28, 40, 45). These include activation of membrane growth factor receptors (i.e., CD40) through mutation or gene amplification, overexpression of growth factors (i.e., CD40L and CD30L), amplification of AKT genes, and activation of intracellular mediators (46, 47). Dal Col et al. (47) found that constitutive activation of AKT in both MCL cell lines and in tumor biopsies was correlated with phosphorylation and inactivation of PTEN. A comprehensive analysis of all these mechanisms will uncover the causes of AKT activation in MCL.

In summary, we show that PIK3CA gene amplification is frequent in MCL and contributes to PI3K/AKT oncogenic activation. Therefore, PIK3CA is an attractive molecular marker and a promising therapeutic target in MCL. Our results, if validated in a second cohort, have important translational implications. Determination of PIK3CA gene and PTEN protein status may have value for patient selection in clinical trials testing PI3K or AKT inhibitors. Future efforts should also focus on the identification of additional genetic changes that lead to AKT activation in MCL.

**Conclusions**

PIK3CA gene amplification is a frequent genetic alteration that contributes to MCL progression. Gain of gene copy number of PIK3CA correlates with increased PIK3CA transcription levels and AKT oncogenic activation. In addition, PIK3CA gene amplification and PTEN protein inactivation were almost mutually exclusive mechanisms of AKT activation. Inhibition of PIK3CA induced increased apoptosis in the MCL cell lines. Our findings are important for two reasons: first, they identify gain of PIK3CA gene copy number as an additional mechanism of AKT activation in MCL, and second, they raise the possibility that PIK3CA can be used as a molecular target in MCL.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**PIK3CA Gene Amplification in MCL**

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Phosphatidylinositol 3'-Kinase Catalytic Subunit α Gene Amplification Contributes to the Pathogenesis of Mantle Cell Lymphoma

Amanda Psyrri, Sotirios Papageorgiou, Elisavet Liakata, et al.


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