Germline *PIK3CA* and *AKT1* Mutations in Cowden and Cowden-like Syndromes

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Cowden syndrome (CS) is a difficult-to-recognize multiple hamartoma syndrome with high risks of breast, thyroid, and other cancers. Germline mutations in *PTEN* on 10q23 were found to cause 85% of CS when accrued from tertiary academic centers, but prospective accrual from the community over the last 12 years has revealed a 25% *PTEN* mutation frequency. PTEN is the phosphatase that has been implicated in a heritable cancer syndrome and subsequently in multiple sporadic cancers and developmental processes. PTEN antagonizes the AKT1/PI3K signaling pathway and has roles in cell cycle, migration, cell polarity, and apoptosis. We report that 8 of 91 (8.8%) unrelated CS individuals without germline *PTEN* mutations carried 10 germline *PIK3CA* mutations (7 missense, 1 nonsense, and 2 indels) and 2 (2.2%) *AKT1* mutations. These mutations result in significantly increased P-Thr308-AKT and increased cellular PIP3. Our observations suggest that *PIK3CA* and *AKT1* are CS susceptibility genes.

Germline mutations in *PTEN* (OMIM 176920) on 10q23 were found in 85% of families with Cowden syndrome (CS) (OMIM 158350) when accrued from tertiary academic centers.[1,2] Prospective accrual from the community over the last 12 years has revealed that 25% of CS cases are due to *PTEN* mutations;[3] 10% of CS individuals without a detected *PTEN* mutation carry germline *SDHx* variants[4] and ~30% have germline *KLLN* (OMIM 612105) hypermethylation.[5] CS is a clinical mimic and difficult to recognize with broad phenotypic presentations and reduced penetrance. In this context, identification of additional predisposition genes would facilitate molecular diagnosis, predictive testing, genetic counseling, and medical management. We created a *PTEN* Cleveland Clinic (CC) Risk Calculator based on prospective accrual of >3,000 CS and Cowden-like (CSL) individuals and multiple logistic regression weighted by neoplasia risk in *PTEN* mutation positive versus individuals without a detected *PTEN* mutation in our cohort compared to that in the general population and by age of neoplasia onset in our cohort.[3] Increasing CC score correlates with higher prior probability of finding *PTEN* mutations. For clinical purposes, we suggest a threshold of 10 (>3%–5% prior probability) for consideration of *PTEN* testing. Based on this rationale, we selected 91 CS/CSL individuals without mutations in *PTEN* or *SDHx*, or hypermethylation of the *KLLN* promoter, and with CC scores ranging from 8 to 54 (adults), with >80% having scores >8 or who met pediatric criteria. We sought to determine whether individuals with high CC scores without alterations in known genes have mutations in genes encoding proteins immediately downstream of *PTEN*.

Germline genomic DNA from 91 unrelated consenting (IRB-8458-PTEN) CS/CSL probands, without *PTEN/SDHx/KLLN* mutations/alterations, were analyzed by Sanger sequencing (ABI3730xl) of *AKT1* (OMIM 164730), *PIK3CA* (OMIM 171834), *PIK3R1* (OMIM 171833), and *PIK3R2* (OMIM 603157) (see Table S1 available online). While no germline mutations were found in *PIK3R1* (NM_181523.2) and *PIK3R2* (NM_005027.2), ten (10.99%) probands were found to carry germline *PIK3CA* and *AKT1* mutations (NM_006218.2, NM_001014431.1) (Table 1; Figure 1; Table S2). None of the mutations were found in 96 population controls, dbSNP, or the available data set in 1000 Genomes Project. To predict the functionality of the germline mutations, we used MutPred software[6] and three-dimensional (3D) protein modeling (Discovery Studio-3.1, Accelrys Inc) of the mutations within their respective domains. The MutPred software, which calculates the probability of a deleterious mutation and corresponding hypothesis of disrupted molecular mechanism, revealed values between 0.46 and 0.88 for our detected variants/mutations. MutPred values between 0.45 and 0.75 predict for benign variations and values >0.75 pathogenic mutations. Three-dimensional modeling revealed that all of the mutations altered polarity and conformation and/or stability of specific domains, leading to or resulting in inappropriate exposure or hiding of key amino acid functional domains. Two mutations in *PIK3CA* c.353G>A and c.1145G>A that result in p.Gly118Asp (MutPred = 0.496) and p.Arg382Lys (MutPred = 0.464) alterations, respectively, had 3D plots showing significant structural alterations (Figures S1 and S2); furthermore, the germline p.Gly118Asp has been reported as a somatic change with functionality in a sporadic malignancy.

PIK3CA encodes p110α, the catalytic subunit of PI3K, which adds a phosphate to phosphatidylinositol-4,5-biphosphate (PIP2) to form phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cellular membrane.[7–10] PIP3 recruits PH domain-containing proteins, e.g., AKT1 to the

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http://dx.doi.org/10.1016/j.ajhg.2012.10.021.

Table 1. CS/CSL Individuals with Germline *AKT1* and *PIK3CA* Mutations

Sample CCF IDs	Gene	Exon	Mutations	MutPred	Gender	Age	CC Score
CCF03121-01-001DG	*AKT1*	2	c.73C>T, p.Arg25Cys	0.879	F	38	8
CCF04333-01-001LM	*AKT1*	12	c.1303A>C, p.Thr435Pro	0.595	F	47	22
CCF03451-01-001DC	*PIK3CA*	2	c.353G>A, p.Gly118Asp	0.496	M	32	12
CCF04880-01-001MQ	*PIK3CA*	2	c.403G>A, p.Glu135Lys	0.677	F	54	13
CCF00102-01-001GG	*PIK3CA*	3	c.652G>A, p.Glu218Lys	0.598	F	44	22
CCF03330-01-001LM	*PIK3CA*	5	c.1066G>A, p.Val356Ile	0.503	F	35	10
CCF02069-01-001CS	*PIK3CA*	5	c.1145G>A, p.Arg382Lys	0.464	M	47	22
CCF04477-01-001FN	*PIK3CA*	9	c.1634A>C, p.Glu545Ala and c.1658_1659 delGTinsC, p. Ser553Thrfs*7	0.769	F	71	13
CCF05779-01-001SC	*PIK3CA*	9	c.1634A>C, p.Glu545Ala and c.1658_1659 delGTinsC p. Ser553Thrfs*7	0.769	F	27	22
CCF00494-01-001WG	*PIK3CA*	11	c.1895T>G, p.Leu632*	N/A	M	59	7

CC Score, *PTEN* Cleveland Clinic Clinical Score for a priori probability of finding *PTEN* mutations. Accession numbers used are NM_001014431.1 (*AKT1*) and NM_006218.2 (*PIK3CA*).

cell membrane.[11] We performed western blot analysis for phospho(P)-AKT1 and p110α in germline protein lysates extracted from CS-derived lymphoblastoid cells from *AKT1* and *PIK3CA* mutation-positive individuals and four controls (Figure 2), using antibodies against P-AKT1^{Ser473} (Epitomics Inc., Burlingame, CA), P-AKT1^{Thr308} (Santa Cruz Biotechnology, CA), p110alpha (Cell Signaling Technology, Danvers, MA), and actin (Santa Cruz Biotechnology). All mutation-positive protein lysates showed a significant increase of P-Thr308-AKT1 levels (Figures 2A and 2B). No obvious differences in P-Ser473-AKT1 were noted between controls and individuals with the mutation, corroborating the specificity of the mutations because Thr308-AKT1, but not Ser473-AKT, is a phosphorylation target of p110α[12]. To assess the impact of *PIK3CA* mutations on PI3K activity, we used immunofluorescence to compare PIP_3 in lymphoblastoid cell lines derived from two individuals that carry germline *PIK3CA* mutations to that from WT individuals (as described,[13] Figure 3). Lymphoblastoid cells were incubated on poly-L-lysine-coated slides for 30 min. Cells were then washed with PBS and then fixed with 100% ice-cold methanol (for 1 min) on slides. Slides were then blocked in 5% goat serum PBS and incubated sequentially with anti-phosphatidylinositol PIP3 antibody (MBL International, Woburn, MA, Cat# D145-3) and Alexa Fluor secondary antibody. Slides were counterstained with DAPI and visualized on a Leica TCS-SP spectral laser scanning confocal microscope. Counterstaining with DAPI revealed increased levels of PIP3 in PIK3CA-p.Glu218Lys and moderately increased levels in PIK3CA-p.Arg382Lys cells compared to wild-type (WT) (Figure 3). Cells bearing PTEN-p.Arg335* served as a positive control (Figure 3). Treating the PTEN-p.Arg335* cells with Wortmannin (PI3K inhibitor) decreased PIP3 levels.

We initially predicted that germline *AKT1* mutations would be more frequent than those in *PIK3CA* based on the report that frequent postzygotic somatic *AKT1* mutations occur in affected tissues of Proteus syndrome, also rarely associated with *PTEN* mutations.[14,15] Instead, we found that 2.2% of CS/CSL probands carried germline *AKT1* mutations (c.73C>T and c.1303A>C, Table 1). The PH-domain p.Arg25Cys germline change has previously been reported as a somatic alteration in noninherited cancers, with insulin-dependent increased kinase activity[16] but abrogation of estradiol-related kinase activity.[17] In vitro insulin exposure results in AKT1-p.Arg25Cys stabilizing p110α and increasing the latter's activity phosphorylating and upregulating AKT1.[16] Our western blot supports this observation, showing tendency toward increased p110α and a significantly increased phosphorylation of AKT1 at Thr308 (Figures 2A and 2B). The

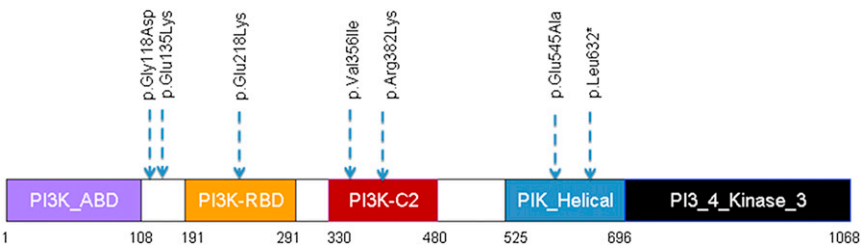

Figure 1. Spectrum of Germline *PIK3CA* Mutations in CS/CSL Individuals without *PTEN* Germline Mutations
A display of amino acid changes that result from different germline *PIK3CA* mutations in CS/CSL individuals (blue arrows) relative to PIK3CA functional domains.

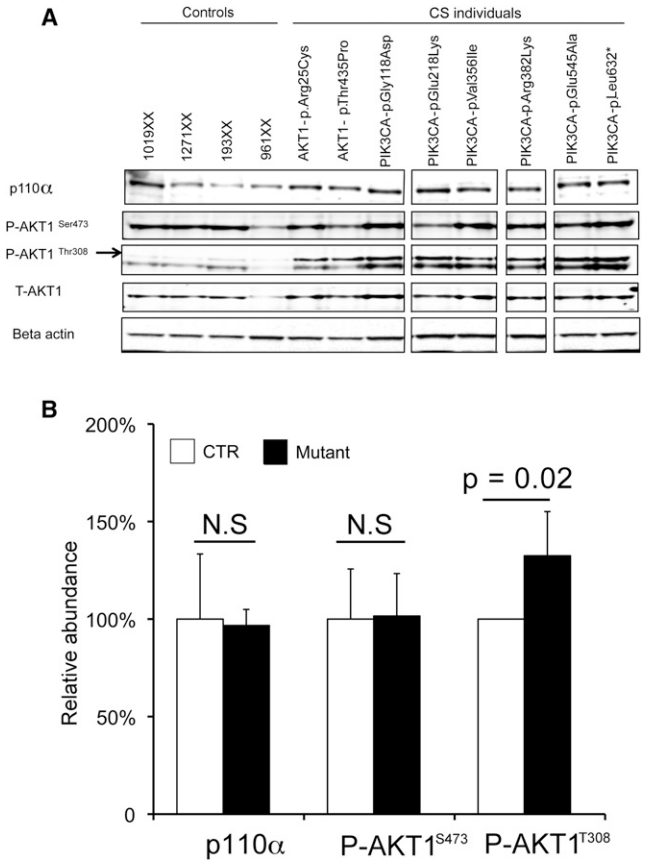

Figure 2. *AKT1* and *PIK3CA* Mutations Result in Upregulation of Phosphorylated P-Thr308-AKT1

(A) Western analysis of peripheral blood protein lysates from CS/CSL individuals with germline *AKT1* and *PIK3CA* mutations. Mutation-positive individuals showed modest increase in p110α levels and marked increased levels of P-Thr308-AKT1 (arrow) but no detectable difference in P-Ser473-AKT1 and total (T)-AKT1 protein levels compared to controls.

(B) Densitometric analysis of specific signals using Image J software. Signals were normalized to actin. A value of 100% was arbitrarily assigned to the ratio obtained in the controls (n = 4) and the relative ratio was made in the individuals with mutations (n = 8). Two-tailed Student's t test was utilized. N.S., not significant.

p.Thr435Pro mutation has not been described before but the end result is increased P-AKT1.

We found that >8.8% CS/CSL probands, without detected *PTEN* germline mutation, have heterozygous germline *PIK3CA* mutations but not in genes encoding the regulatory subunits of PI3K, *PIK3R1/PIK3R2* (Table 1). Somatic mutations in these three genes have been amply described in solid tumors, including those of the breast, thyroid, and endometrium[18–20]. Overall, the somatic mutational spectra and the germline spectra in *PIK3CA* appear different, with the majority of CS/CSL mutations within the C2-domain. C2 is responsible for p110α recruitment to cellular membranes. C2 mutations have been shown in vitro to alter charge or conformation enhancing recruitment of the mutant-p110α to cell membranes.[21] Corroborating this, western analysis reveals slightly increased p110α and markedly increased P-Thr308-AKT (Figure 2A). Recently, postzygotic and mosaic mutations in the genes encoding various proteins in the AKT/PI3K signaling pathway have been described in rare nonneoplasia hemihypertrophy-segmental overgrowth syndromes.[13,22,23] Note that C2-domain *PIK3CA* mutations are prominent in megalencephaly-capillary malformation syndrome as well. Among all these syndromes, including CS/CSL, involvement of the brain and vascular malformation are not infrequent. Just as we proposed the term PTEN hamartoma tumor syndrome (PHTS) for any individual irrespective of clinical presentation carrying a germline *PTEN* mutation, we would like to propose the term PTEN/AKT/PI3K-opathy to encompass this broad range of phenotypes.

In summary, 11% of Cowden and Cowden-like syndrome individuals without detected *PTEN/SDHx/KLLN* mutation/alteration carry germline gain-of-function *AKT1* or *PIK3CA* mutations. Together, the *PIK3CA* and *AKT1* pathways represent a novel driver of predisposition to hamartoma-neoplasia syndromes that may have diagnostic, genetic counseling, and therapeutic implications.

Supplemental Data

Supplemental Data includes two figures and two tables and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

We are deeply grateful to all of our research participants and their families, clinicians, and genetic professionals who have contributed one way or the other to our studies over the many years, especially the last 6 years. This study is funded, in part, by NCI grant P01CA124570 and the American Cancer Society Clinical Research Professorship (both to C.E.). C.E. is the Sondra J. and Stephen R. Hardis Chair in Cancer Genomic Medicine at the Cleveland Clinic.

Received: June 23, 2012
Revised: October 5, 2012
Accepted: October 29, 2012
Published: December 13, 2012

Web Resources

The URLs for data presented herein are as follows:

OMIM, www.omim.org
PTEN Cleveland Clinic (CC) Score and Risk Calculator, http://www.lerner.ccf.org/gmi/ccscore

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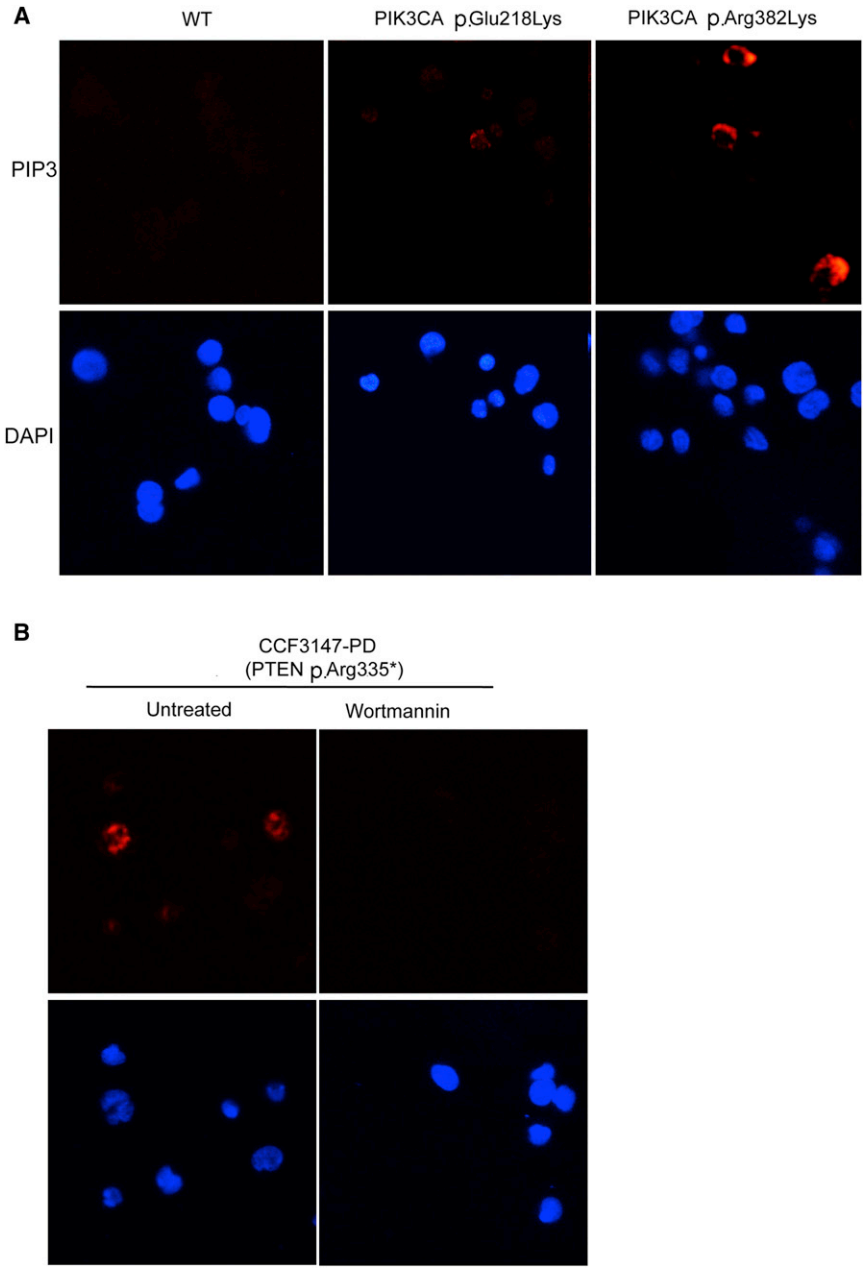

Figure 3. Increased PIP_3 Levels in Lymphoblastoid Cell Lines (LCLs) Derived from *PIK3CA* Mutation-Positive Individuals
(A) Indirect immunofluorescence staining of PIP3 (red) and DAPI (blue) in LCLs derived from an unaffected control (WT) and two CS individuals with *PIK3CA* germline mutations.
(B) LCLs isolated from a CS individual harboring a *PTEN* germline mutation (p.Arg335*) that were either treated or not treated with the PI3K inhibitor, Wortmannin (100 nM for 30 min), serve as positive control (left) and negative control (right) for PIP3 levels, respectively.

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