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## A unique spectrum of somatic *PIK3CA* (p110 $\alpha$ ) mutations within primary endometrial carcinomas

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### Abstract

**Purpose**—The goal of this study was to comprehensively define the incidence of mutations in all exons of *PIK3CA* in both endometrioid endometrial cancer (EEC) and non-endometrioid endometrial cancer (NEEC).

**Experimental design**—We resequenced all coding exons of *PIK3CA* and *PTEN*, and exons 1 and 2 of *KRAS*, from 108 primary endometrial tumors. Somatic mutations were confirmed by sequencing matched normal DNAs. The biochemical properties of a subset of novel *PIK3CA* mutations were determined by exogenously expressing wildtype and mutant constructs in U2OS cells and measuring levels of AKT<sup>Ser473</sup> phosphorylation.

**Results**—Somatic *PIK3CA* mutations were detected in 52.4% of 42 EECs and 33.3% of 66 NEECs. Half (29 of 58) of all nonsynonymous *PIK3CA* mutations were in exons 1–7 and half were in exons 9 and 20. The exons 1–7 mutations localized to the ABD, ABD-RBD linker and C2 domains of p110 $\alpha$ . Within these regions, Arg88, Arg93, Gly106, Lys111, Glu365, and Glu453, were recurrently mutated; Arg88, Arg93 and Lys111 formed mutation hotspots. The p110 $\alpha$ -R93W, -G106R, -G106V, -K111E, -delP449-L455, and -E453K mutants led to increased levels of phospho-AKT<sup>Ser473</sup> compared to wild-type p110 $\alpha$ . Overall, 62% of exons 1–7 *PIK3CA* mutants and 64% of exon 9–20 *PIK3CA* mutants were activating; 72% of exon 1–7 mutations have not previously been reported in endometrial cancer.

**Conclusions**—Our study identified a new subgroup of endometrial cancer patients with activating mutations in the amino-terminal domains of p110 $\alpha$ ; these patients might be appropriate for consideration in clinical trials of targeted therapies directed against the PI3K pathway.

### Keywords

Endometrial; cancer; *PIK3CA*; mutation; spectrum

## INTRODUCTION

Endometrial cancer is the eighth leading cause of cancer-related death among American women (1). At presentation, the vast majority of tumors are endometrioid endometrial cancers (EECs), which are estrogen-dependent tumors that may be preceded by endometrial intraepithelial neoplasia, a premalignant outgrowth from hormonally-induced, benign endometrial hyperplasia (2). Most EECs are detected at an early stage when surgery is an effective form of treatment (3). The clinical management of recurrent and advanced disease includes surgery, followed by chemotherapy or radiotherapy alone or in combination (4). However, the prognosis for women with recurrent or advanced stage EECs is relatively poor (5,6) and alternative therapeutic options are needed.

In contrast to EECs, non-endometrioid endometrial cancers (NEECs) are high-grade, estrogen-independent tumors that arise from the atrophic endometrium in postmenopausal women (7). NEECs are clinically aggressive and have a significantly worse prognosis than EECs even when corrected for tumor stage (5,6). Current therapeutic strategies to manage NEECs are variable but generally include surgery and adjuvant therapy, even in cases of early-stage disease (4,8). Although NEECs represent a minority (5–10%) of cases at presentation, they account for a disproportionate number of all endometrial cancer-related deaths (5). Thus, there is a critical need for improved therapeutic options for patients with this tumor subtype.

The PI3K $\alpha$ -mediated signal transduction pathway is an important therapeutic target for molecularly-defined subsets of cancer (9). PI3K $\alpha$  is a heterodimeric protein complex comprised of the catalytic p110 $\alpha$  subunit and the regulatory p85 $\alpha$  subunit, which are encoded by the *PIK3CA* and *PIK3RI* genes respectively (9). Inappropriate activation of PI3K $\alpha$ -mediated signaling is frequent in human cancers, resulting in increased AKT-dependent or AKT-independent signaling and leading to increased cell proliferation, growth, survival and migration (10,11). The pathway is antagonized by the activity of the PTEN phosphatase (12–14).

A number of pharmacological agents targeting components of the PI3K signal transduction pathway have been developed (9,15). These include PI3K inhibitors, AKT inhibitors, and inhibitors of downstream effectors such as mTOR (mammalian target of rapamycin), a serine-threonine kinase that mediates PI3K-AKT signaling (9). A number of PI3K-pathway inhibitors have already entered clinical trials, including trials to assess efficacy in endometrial cancer patients (16–19).

Common mechanisms of PI3K $\alpha$  activation in tumorigenesis are the acquisition of somatic gain-of-function mutations within *PIK3CA* (20), or loss of *PTEN* activity resulting from mutations or gene deletion (12,14). Early studies of colorectal, breast, ovarian, and bladder cancers, in which all coding exons of *PIK3CA* were sequenced, revealed that ~80% of all *PIK3CA* mutations occurred within exons 9 and 20, which encode the C-terminal helical and kinase domains of p110 $\alpha$  (20–22). A much smaller fraction (~20%) of *PIK3CA* mutations in these tumors were within exons 1–7, which encode the N-terminal domains of p110 $\alpha$  including the p85/adaptor-binding domain (ABD) and the protein kinase-C homology 2 (C2) domain (23).

*PIK3CA* exons 9 and 20 mutations are present in 10%–36% of sporadic EECs (24–30) and 15%–21% of NEECs (31,32). However, most resequencing studies of *PIK3CA* in sporadic endometrial cancer have been confined to exons 9 and 20 because these two exons encompass >80% of mutations in other tumor types (20). Consequently, the incidence of mutations in other exons of *PIK3CA* in endometrial tumors has not been rigorously defined. Interestingly however, one study noted a disproportionate number of exon 1 mutations (4 of

6 mutations) in a small series of endometrial cancer cell-lines (33). Another study of primary sporadic EECs observed that 4 of 9 *PIK3CA* mutations in EECs were in exon 1 (34). Because the frequency of amino terminal p110 $\alpha$  mutants relative to carboxy-terminal domain mutants was relatively high in these two studies, we hypothesized that endometrial carcinomas might have a different spectrum of *PIK3CA* mutations compared with other tumor types. Given the therapeutic importance of altered PI3K $\alpha$ -mediated signaling, we therefore sought to rigorously define the overall frequency and distribution of somatic *PIK3CA* mutations in a large series of 108 primary endometrial cancers comprised of EECs and NEECs.

Here we show that in both EECs and NEECs, *PIK3CA* mutations are as frequent within exons 1–7 as within exons 9–20. Almost all exon 1–7 mutations clustered within three specific regions of p110 $\alpha$ , the ABD, the ABD-RBD linker and the C2 domain. Our study revealed three major mutational hotspots at amino acids 88, 93 and 111 within the ABD and its adjacent linker. We extended our study to investigate the biochemical properties of ten previously uncharacterized *PIK3CA* mutants within exons 1–7. Six of the ten mutations tested encoded gain-of-function p110 $\alpha$  mutants that increased levels of phospho-AKT(Ser473), compared to wildtype p110 $\alpha$ . Overall, 62% of all mutations in exons 1–7 encoded gain-of-function mutants of p110 $\alpha$ . We further show that, like *PIK3CA* exon 9–20 mutations, mutation in exon 1–7 can co-exist with *PTEN* and *KRAS* mutations; 93% of EECs and 38% of NEECs in our study have a mutation in at least one of these three genes. Taken together, our genetic and biochemical data show that endometrial cancers, unlike other tumors, have a high frequency of somatic activating mutations within the ABD, ABD-RBD linker, and C2 domains of p110 $\alpha$ . Our findings have potential clinical implications because the mutational status of *PIK3CA* can guide patient stratification for genotype-directed clinical trials of rationally designed therapies targeting the PI3K pathway.

## MATERIALS AND METHODS

### Clinical material

Snap-frozen primary tumor tissues, corresponding hematoxylin and eosin (H&E)-stained tumor sections, and matched normal tissues, were obtained from the Cooperative Human Tissue Network, which is funded by the National Cancer Institute, or from the Biosample Repository at Fox Chase Cancer Center, Philadelphia PA. Matched tumor and normal DNAs for six cases were purchased from Oncomatrix. Tumor specimens were collected at surgical resection, prior to treatment. Tumors were selected for endometrioid (n=42), serous (n=46) and clear cell (n=20) histologies. A histological classification was rendered based upon the entire specimen at time of diagnosis. Matched normal tissues were uninvolved reproductive tissue or whole blood. All tissues, and accompanying clinicopathological information, were anonymized and collected with appropriate IRB approval. A pathologist compared the H&E section to the original classification to verify that it was representative histologically, and to delineate regions of tissue comprised of >70% tumor cells for macrodissection.

### DNA extraction and identity testing

Genomic DNA was isolated from macrodissected tumor tissues and normal tissues using the PUREGENE kit (Qiagen). To confirm that tumor-normal pairs were derived from the same individual, each DNA sample was typed using the Coriell Identity Mapping kit (Coriell). Genotyping fragments were resolved on an ABI-3730xl DNA analyzer (Applied Biosystems) and scored using GeneMapper software.

## Mutational analysis by nucleotide sequencing

Primers were designed to PCR amplify all coding exons of *PIK3CA* and *PTEN*, and exons 1 and 2 of *KRAS* (Supplementary Table 1) from tumor DNAs. *PIK3CA* exons 9–13 primers were designed to avoid amplification of a pseudogene. PCR conditions are available on request. PCR amplicons were resolved by gel electrophoresis, purified by exonuclease I (Epicentre Biotechnologies) and shrimp alkaline phosphatase (USB Corporation) treatment, and bidirectionally sequenced using Big Dye Terminator v.3.1 (Applied Biosystems). Sequencing products were ethanol precipitated and resolved on an ABI-3730xl DNA analyzer (Applied Biosystems). Tumor sequences were aligned to a reference sequence using Sequencher software (Gene Codes Corporation) and visually inspected to identify variant positions. Nucleotide variants absent from dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) were confirmed by sequencing an independently generated PCR product. Resequencing of matched normal DNA distinguished somatic mutations from novel germline polymorphisms.

## Statistical analyses

All comparisons between groups were performed using a 2-tailed Fisher's Exact test of significance.

## Mammalian cell culture

The U2OS human osteosarcoma cell line was maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen), at 37°C, in a humidified atmosphere.

## Generation of expression constructs

A baculovirus expression construct containing the full-length, wild-type, *PIK3CA* cDNA cloned into the pFastBac vector (Invitrogen), was a kind gift from Dr. Yardena Samuels (NHGRI/NIH). This construct was used as a template to generate a series of *PIK3CA* mutant constructs by site directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions. The mutations created corresponded to the R93Q, R93W, G106R, G106V, K111E, E453A, E453K, E365K, G364R, H1047R, and delP449-455 mutants of p110 $\alpha$ . Wild-type and mutant *PIK3CA* inserts were excised using *Bam*HI and *Hind*III and subcloned into the FLAG-tagged pCMV-3Tag-1A expression vector (Agilent Technologies). Sanger sequencing was used to confirm the integrity of the cloning sites and the *PIK3CA* inserts.

## Transfections, and western blotting

U2OS cells were transfected with vector, wild-type, or mutant expression constructs using FuGENE-6 transfection reagent (Roche), according to the manufacturer's protocol. Stably transfected cells were selected in the presence of 1000 $\mu$ g/ml G418 (Invitrogen). For western blots, pools of stably selected cells were serum starved in DMEM containing 0.5% FBS for 15hr and lysed in lysis buffer (1% TritonX-100, 150mM NaCl, 50mM Tris-HCL pH 7.4, 1mM EDTA, 1mM Na-orthovanadate, 10mM NaF, 1X protease inhibitor cocktail (Roche)). Lysates were centrifuged at 4°C for 10min at 12,000xg to remove insoluble cellular debris and denatured at 95°C in 2X SDS sample buffer (Sigma) for 5min prior to electrophoresis. Denatured proteins (20–40 $\mu$ g) were resolved with SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were blocked in 5% milk/TBST for 30min at room temperature and subsequently blotted with appropriate primary and HRP-conjugated secondary antibodies: FLAG-M2 (Stratagene),  $\beta$ -Actin (Sigma), phospho-AKT<sup>Ser473</sup>, phospho-AKT<sup>T308</sup>, total AKT (Cell Signaling), Goat anti-mouse HRP (Santa Cruz), and Goat anti-Rabbit HRP (Cell Signaling). Immunoreactive proteins were visualized with

enhanced chemiluminescence (Pierce) and quantitated with ImageJ software (National Institutes of Health, Bethesda MD). Normalized band intensities for FLAG-, phospho-AKT<sup>Ser473</sup>, and total AKT were determined by comparison to respective  $\beta$ -Actin band intensities. The average ratio of normalized phospho-AKT to FLAG was calculated for each construct and used to plot the fold change in phospho-AKT<sup>Ser473</sup> levels for mutants compared to wild-type. For quantitation, western blots were repeated in triplicate.

## RESULTS

### Somatic mutations in exons 1–7 of *PIK3CA* are frequent in endometrial cancer

Resequencing exons 1–20 of *PIK3CA* from a series of 108 primary endometrial tumors revealed somatic *PIK3CA* mutations in 52.4% (22 of 42) of EECs and 33.3% (22 of 66) of NEECs (Tables 1 and 2), a difference that approached statistical significance ( $P=0.07$ ). Among NEECs, the frequency of *PIK3CA* mutations in serous (34.7%, 16 of 46) and clear cell tumors (30%, 6 of 20) was comparable. We observed no significant correlations between *PIK3CA* mutations and histologic grade or FIGO stage of tumors (Supplementary Tables 2 and 3). In both EECs and NEECs *PIK3CA* mutations were detected at all stages, including stage 1A tumors. Within the EECs, there was no evident association between tumor grade and *PIK3CA* mutation status.

Of 62 somatic *PIK3CA* mutations detected, 58 were exonic and four were intronic (Tables 1 and 2). All exonic mutations were nonsynonymous. The majority (86.2%, 50 of 58) of nonsynonymous mutations were either known gain-of-function mutations (48.3%, 28 of 58), or were of unknown functional significance but previously observed in other cancers (37.9%, 22 of 58) suggesting they are likely to be functionally significant (10,33,35–37). A minority (15.5%, 9 of 58) of nonsynonymous mutations was novel. A review of the COSMIC mutation database and published literature revealed that 26 of the 58 (44%) exonic mutations identified in this study have not previously been reported in endometrial cancer (Tables 1 and 2) (33,35).

Fifty-percent (29 of 58) of all exonic *PIK3CA* mutations localized within exons 1–7. The remaining 50% of exonic mutations localized within exons 9–20. The high frequency of *PIK3CA* exon 1–7 mutations was observed for all three histotypes; exon 1–7 mutations constituted 41% (12 of 29) of *PIK3CA* mutations in endometrioid tumors, 50% (10 of 20) of *PIK3CA* mutations in serous tumors, and 78% (7 of 9) of *PIK3CA* mutations in clear cell tumors. The frequency of mutations in exons 1–7 was not significantly different between NEECs (58.6%, 17 of 29) and EECs (41%, 12 of 29), ( $P=0.29$ ). However, the overall incidence of mutations in exons 1–7 of *PIK3CA* in this series of 108 endometrial tumors (50%, 29 of 58) was statistically significantly more frequent than others have observed for colorectal cancer (18.5%, 15 of 81;  $P=0.0001$ ), breast cancer (10.7%, 3 of 28;  $P=0.0003$ ) or bladder cancer (2.8%, 1 of 36;  $P<0.0001$ ) (20–22).

A subset of mutated tumors (29.5%, 13 of 44) had multiple nonsynonymous *PIK3CA* mutations. Four tumors had co-existing mutations in exons 1–7; six tumors had coexisting mutations in exons 1–7 and exons 9–20; and three tumors had co-existing mutations in exons 9–20. Ten of the 13 tumors had mutations in at least two different domains of p110 $\alpha$ .

### Mutations in exons 1–7 of *PIK3CA*

Among the 108 endometrial tumors in this study, 24 (22%) had at least one somatic mutation in exons 1–7 of *PIK3CA*. Of the 29 individual exon 1–7 mutations, 21 (72%) have not previously been observed in sporadic endometrial cancer (Tables 1 and 2); only the activating R88Q, R108H, and E365K mutations have previously been reported (33–35).

The vast majority (28 of 29, 96.5%) of *PIK3CA* exon 1–7 mutations in our study localized to the ABD, ADB-RBD linker, and C2 domains of p110 $\alpha$  (Figure 1A). Within these domains, six amino acid residues, Arg<sup>88</sup> (R88), Arg<sup>93</sup> (R93), Gly<sup>106</sup> (G106), Lys<sup>111</sup> (K111), Glu<sup>365</sup> (E365), and Glu<sup>453</sup> (E453), were recurrently mutated. Three of these recurrently mutated residues, R88, R93, and K111, formed hotspots that together accounted for 22% (13 of 58) of all exonic mutations in this study.

Among the 29 individual mutations in exons 1–7, 20 mutations were unique. Five of 20 (25%) unique mutations, namely R88Q, K111N, R108H, E365K, and C420R, are known to encode gain-of-function mutants of p110 $\alpha$  (33,37). The remaining 15 unique mutations have not been functionally characterized. Of these uncharacterized mutations, nine (E81K, R93W, R93Q, G106V, delE109, K111E, G118D, V344A, and E453K), were either recurrent within this study, or were recurrent between this study and other studies (35), suggesting that they might confer a selective advantage in tumorigenesis. The six remaining uncharacterized mutations were novel. Two of the novel mutations (delL15\_V22insHPI and G106R) localized to the ABD of p110 $\alpha$ , one (A222V) localized to the RBD, and three (G364R, delP449-L445, E453A) localized to the C2 domain. Three novel mutations (delL15\_V22insHPI, G106R, delP449-L445, E453A) involved amino acids that undergo a different mutation here or in other studies (31).

### Mutations in exons 9–20 of *PIK3CA*

Fifty-percent (29 of 58) of all nonsynonymous *PIK3CA* mutations localized within exons 9–20. Of the 29 individual mutations in exons 9–20, 18 (62%) are known to encode activating mutants of p110 $\alpha$ . Activating mutations at codons 542, 545, and 1047, known mutational hotspots in p110 $\alpha$ , accounted for 25.8% (15 of 58) of all exonic mutations. The frequency of kinase domain mutations (32.7%, 19 of 58) was somewhat greater than the frequency of helical domain mutations (17.2%, 10 of 58), but this did not reach statistical significance ( $P=0.085$ ). Only five (17%) mutations within exons 9–20 (F667L, T1025N, M1040L, M1004F, and Q546P), have not previously been observed in endometrial tumors (35). Of these four mutations, one (Q546P) is a known gain-of function mutation that is present in seven tumors from other anatomical sites (35,37).

### Patterns of *PIK3CA*, *PTEN*, and *KRAS* mutations in EECs and NEECs

Previous studies of sporadic EECs have shown that mutations in exons 9 and 20 of *PIK3CA* can coexist with mutations in *PTEN* and *KRAS*, two additional genes within the PI3K-mediated signal transduction pathway (25,26,29,38). Because our findings revealed a new subset of endometrial cancer patients with mutations in exons 1–7 of *PIK3CA*, we extended our study to *PTEN* and *KRAS* in order to comprehensively determine the incidence and pattern of *PIK3CA-PTEN-KRAS* mutations in both EECs and NEECs.

Overall, 93% (39 of 42) of EECs and 38% (25 of 66) of NEECs in this study had a somatic mutation in at least one of these three genes (Figure 1B). Somatic *PTEN* mutations were present in 78.6% (33 of 42) of EECs and 10.6% (7 of 66) of NEECs ( $P < 0.0001$ ) (Supplementary Figure 1 and Supplementary Table 4). Somatic *KRAS* mutations were detected in 42.8% (18 of 42) of EECs and 4.5% (3 of 66) of NEECs ( $P < 0.0001$ ) (Supplementary Table 5). The frequencies of *PTEN* and *KRAS* mutations observed in EECs are higher than published data (24, 25), whereas the frequency of such mutations in NEECs is within the range of published data (35).

All permutations of co-existing *PIK3CA*, *PTEN*, and *KRAS* mutations were observed but their relative frequencies differed between EECs and NEECs (Figure 1B). The vast majority (64.2%, 27 of 42) of EECs had mutations within at least two of the three genes. In contrast,

the most frequently observed mutation pattern in NEECs was single mutants of *PIK3CA*, which accounted for 24.2% (16 of 66) of all cases.

In a previous study of endometrial cancers there was a tendency for *PIK3CA* exons 9 and 20 mutations to be more frequent in *PTEN*-mutant tumors than in *PTEN* wildtype tumors (26). Here, we evaluated the pattern of *PIK3CA* exons 1–20 mutations and *PTEN* mutations within EECs wherein the incidence of *PTEN* mutations was 7.4-fold higher than in NEECs. We observed no significant difference in the frequency of *PIK3CA* mutations among *PTEN* mutant EECs (51.5%, 17 of 33) and *PTEN* wild-type EECs (55.5%, 5 of 9) ( $P=1.0$ ).

*PIK3CA* and *KRAS* mutations coexisted in 19% (8 of 42) of EECs and in 1.5% (1 of 66) of NEECs in our study. Given the higher frequency of *KRAS* mutations in EECs than in NEECs in this study, we evaluated *PIK3CA* and *KRAS* mutation patterns in EECs. We observed no significant difference in the frequency of *KRAS* mutations between *PIK3CA* wild-type EECs and *PIK3CA* mutant EECs (50% (10 of 20) versus 36.4% (8 of 22) respectively) ( $P=0.53$ ). Although there was a tendency for *KRAS* mutations to be present more often in *PTEN* mutant EECs (48.5%, 16 of 33) than in *PTEN* wild-type EECs (22%, 2 of 9 cases), the difference was not statistically significant ( $P=0.26$ ).

### Functional characterization of ABD, ABD-RBD, and C2 domain mutants

Because we observed that *PIK3CA* exon 1–7 mutations were significantly higher in our series of endometrial tumors than in other tumor types, we sought to determine the functional consequences of a subset of exon 1–7 mutations identified here. Eight of the 15 uncharacterized mutations in these exons were distributed among four recurrently mutated residues (R93, G106, K111, E453) of p110 $\alpha$  in our tumor series. We hypothesized that these particular mutations might be functionally significant. We therefore sought to determine the functional significance of the R93W, R93Q, G106R, G106V, K111E, E453A, E453K, delP449-L455 mutants, that together encompass the four recurrently mutated amino acids, as well as the V344A and G364R mutants within the C2 domain.

We examined the ability of each of the p110 $\alpha$  mutants to phosphorylate AKT on Serine-473 (Ser<sup>473</sup>) in stably transfected U2OS (osteosarcoma) cells. We chose U2OS cells because they have low endogenous levels of phospho-AKT, and because they have previously been used to evaluate the activity of other p110 $\alpha$  mutants (33). Two known gain- of-function mutants, p110 $\alpha$ -E365K (a C2 domain mutant) and p110 $\alpha$ -H1047R (a kinase domain mutant), were included as positive controls for AKT activation (10,33).

Under low serum conditions, we observed that the exogenous expression of six mutants, p110 $\alpha$ -R93W, -G106R, -G106V, -K111E, -delP449-L455, and -E453K, led to increased levels of phospho-AKT<sup>Ser473</sup> compared to the wild-type p110 $\alpha$  (Figure 2A and 2B). These six mutations account for 27% (8 of 29) of *PIK3CA* exon 1–7 mutations observed in our study. Interestingly, we observed differences in the level of AKT phosphorylation associated with these mutations. Expression of the p110 $\alpha$ -G106R, -G106V and -delP449-455 mutants led to higher levels of phospho-AKT<sup>Ser473</sup> than the p110 $\alpha$ -H1047R kinase domain mutant. In contrast, the p110 $\alpha$ -R93W, -K111E, and -E453K mutants showed a similar level of phospho-AKT<sup>Ser473</sup> as the p110 $\alpha$ -E365K C2-domain mutant, but lower level phospho-AKT<sup>Ser473</sup> levels than the p110 $\alpha$ -H1047R kinase domain mutant. Phosphorylation on AKT<sup>Thr308</sup> showed the same pattern as AKT<sup>Ser473</sup> (Figure 2).

## DISCUSSION

Here we show that endometrial cancers have unique, and tissue-specific, spectrum of somatic *PIK3CA* mutations. Unlike other tumor types, in which the majority of *PIK3CA*

mutations are within exons 9–20, endometrial cancers display a high frequency of mutations in exons 1–7 of *PIK3CA* as well as in exons 9–20. Over 60% of the somatic mutations in exons 1–7 were activating. The pattern of *PIK3CA* mutations that we observed in endometrial cancers is highly statistically significantly different from that of colorectal cancer, breast cancer and bladder cancer, three tumor types for which *PIK3CA* has been comprehensively resequenced (20–22).

Importantly, our genetic and functional data reveal a new subgroup of endometrial cancer patients who have activating somatic mutations within *PIK3CA*. Had only exons 9 and 20 been sequenced in this study, we would have missed the 16% of NEECs (11 cases) and 16% of all EECs (7 cases) that have *PIK3CA* mutations exclusively in exons 1–7; of these cases at least 8 NEECs and 5 EECs have an activating mutation in exons 1–7. An additional 3% of NEECs (2 cases) and 9% of EECs (4 cases) would have incompletely genotyped since they have mutations in both exons 1–7 and exon 9–20.

Exons 1–7 of *PIK3CA* encode the ABD, RBD, and C2 domains of p110 $\alpha$  whereas exons 9–20 encode the helical and kinase domains. Recent structural studies of p110 $\alpha$  in complex with p85 $\alpha$  have provided critical insights into the distinct properties of the p110 $\alpha$  domains (23,39,40). The ABD of p110 $\alpha$  forms an interface with both the iSH2 domain of p85 $\alpha$  and it also has complex interactions with the first alpha helix of the p110 $\alpha$  ABD-RBD linker region as well as the first alpha helix of the kinase domain of p110 $\alpha$  (40). The C2 domain of p110 $\alpha$  mediates binding to the cell membrane, the kinase domain of p110 $\alpha$  and the iSH2 domain of p85 $\alpha$ . The helical domain acts as a scaffold for the assembly of all other p110 $\alpha$  domains. The catalytic activity of p110 $\alpha$  resides within the kinase domain.

Here we found that almost all mutations in exons 1–7 localized within the ABD, the ABD-RBD linker region, and the C2 domain of p110 $\alpha$  whereas very few mutations localized within the RBD, which binds RAS. This pattern is reminiscent of the distribution of rare exon 1–7 mutations that have been reported in colorectal, breast, and bladder cancers (20). As was previously shown for *PIK3CA* exons 9–20 mutations (26), we found that *PIK3CA* exons 1–7 mutations could co-exist with *PTEN* and *KRAS* mutants in both EECs and NEECs.

The high frequency, and non-random distribution of amino terminal p110 $\alpha$  mutants in EECs and NEECs infers that there is a selective advantage to mutationally disrupting the ABD, the ABD-RBD linker, and the C2 domain of p110 $\alpha$  in endometrial carcinomas. Consistent with this idea, 62% of the 29 individual mutations we found in exons 1–7 of *PIK3CA*, encode gain-of-function mutants of p110 $\alpha$ ; eight mutations were shown in this study to be gain-of-function mutants that lead to increased levels of phospho-AKT<sup>Ser473</sup>, and ten additional mutations were previously shown by others to be gain-of-function mutations (33,37).

Approximately one-third of all nonsynonymous mutations present among the 108 endometrial tumors in this study localized within the ABD and proximal ABD-RBD linker region. Strikingly, within these two regions Arg88, Arg 93, and Lys111 residues formed mutational hotspots that accounted for 21% (6 of 29) of all mutations in EECs and 24% (7 of 29) of all mutations in NEECs in our tumor series.

Each of the mutations at Arg88 resulted in an amino acid substitution of arginine for glutamine (R88Q), a known gain-of-function mutant associated with increased AKT activation *in vitro* (33). Our finding that R88Q is a hotspot in endometrial cancer confirms previous observations by Oda *et al.*, and Dutt *et al.*, in which R88Q constituted 40% (6 of 15) of *PIK3CA* mutations present among 53 endometrial tumors and cell-lines (33,34). Arg88 lies on a highly conserved surface of the ABD (40) and it forms a hydrogen bond with Asp746 in the kinase domain of p110 $\alpha$  (23). It has therefore been proposed that



mutations at Arg88 might disrupt this interaction resulting in an altered kinase domain conformation and increased enzymatic activation of PI3K (23).

Arg93 (R93) formed a second hotspot within the ABD in our tumor series. Two different mutations were found at this residue, R93W and R93Q. Here, we characterized these two mutants functionally and showed that R93W mutation is a gain-of function mutant that leads to increased phosphorylation of AKT on serine 473. In contrast, we observed no evidence for an increase in AKT phosphorylation associated with the R93Q mutant. This was somewhat unexpected because R93Q was mutated in three different endometrial tumors in our study, strongly suggesting that it would have a selective advantage. Interestingly however, each of the three tumors that harbored the R93Q mutant also had at least one other *PIK3CA* mutation in a different domain of p110 $\alpha$  (either T1025N, H1047Y/K111N, or A1066V), whereas tumors with the activating the R93W mutant had no other p110 $\alpha$  mutation. Our observation that R93Q always occurs as a “double” or “triple” mutant could be functionally relevant because Zhao and Vogt (41) have shown that two mutations occurring in different domains of p110 $\alpha$  can functionally synergize and activate PI3K more potently than either mutation alone. We therefore speculate that the p110 $\alpha$ -R93Q mutant might be only weakly activating by itself, below the level of detection in the assays performed here, but that it cooperates with kinase domain mutations to synergistically activate PI3K. Future studies examining the combinatorial effects of the R93Q and its co-occurring mutations will be required to test this hypothesis. However, consistent with the idea that mutations in different domains of p110 $\alpha$  can functionally co-operate, the vast majority of endometrial tumors that had two or more *PIK3CA* mutations in this study had mutations in different domains of p110 $\alpha$ .

In addition to R88 and R93 hotspots, another residue in the ABD, at position 106 (G106), was recurrently mutated in endometrial cancer. We found that both mutations at this site (G106R and G106V) were gain-of-function mutants that increased AKT phosphorylation. It is currently unclear how mutations at this residue affect p110 $\alpha$  activity since structural studies have not revealed a specific interaction mediated by residue 106 (23).

The third mutation hotspot in the amino terminus of p110 $\alpha$  occurred at lysine 111 (K111). One endometrial tumor had a K111N mutation and two additional endometrial tumors had a K111E mutation. Here we showed that the K111E mutant is activating, leading to an increase in AKT phosphorylation compared to wildtype p110 $\alpha$ . We observed that the level of phospho-AKT associated with the K111E was lower than for the H1047R mutant. This observation is consistent with the findings of Gymnopoulous et al., that the K111N mutant of p110 $\alpha$  is more weakly activating than the H1047R kinase domain mutant (37).

The C2 domain of p110 $\alpha$  harbored 13% of all nonsynonymous *PIK3CA* mutations among the endometrial tumors analyzed in this study. Of the eight individual mutations in the C2 domain, three mutations (E365K in 2 cases, and C420R in one case) were previously shown to be activating (33,37). Here we tested the functional consequences of the other five C2 domain mutants that had not been previously characterized. We showed that the delP449-L455 and E453K mutants were activating. Both of these mutants increased AKT phosphorylation at levels similar to, or greater than, the strongly activating H1047R kinase domain mutant. Both p110 $\alpha$ -Glu453 and the adjacent residue Glu454, form hydrogen bonds with p85 $\alpha$ -Glu348 (39). It is therefore likely that the delP449-L455 and E453K mutants disrupt this interaction thus leading to increased catalytic activity. Interestingly we observed that the p110 $\alpha$ -delP449-L455 deletion mutant led to much higher levels of phosphorylated AKT than the p110 $\alpha$ -E453K point mutant. We speculate that higher level of AKT activation seen with the p110 $\alpha$ -delP449-L455 deletion mutant might reflect an additive effect of mutating the p110 $\alpha$ -Glu453 and Glu454, both of which form hydrogen bonds with p85 $\alpha$ ,

whereas the E453K point mutant affects only one of these residues. Although we found no convincing biochemical evidence that the p110 $\alpha$ -V344R, -G364R, and -E453A C2-domain mutants were activating as single mutants, it remains possible that they might contribute to endometrial tumorigenesis via AKT-independent mechanisms (11), or, in the case of p110 $\alpha$ -V344R and p110 $\alpha$ -G364R, which co-occur with other *PIK3CA* mutations, by co-operating with other p110 $\alpha$  mutants. Alternatively, these mutants might be bystander mutations that have no selective advantage to tumorigenesis.

In conclusion, our findings revealed a distinct subgroup of endometrial cancer patients with somatic activating mutations in the amino terminus of p110 $\alpha$  in their tumors. Molecular alterations in the PI3K pathway can point to subgroups of cancer patients who might benefit clinically from rationally designed therapies that target the PI3K signal transduction pathway. Therefore, our findings have potential clinical implications suggesting the need to comprehensively evaluate all coding exons of *PIK3CA* to capture the most appropriate endometrial cancer patients for inclusion in genotype-directed trials of therapeutic agents targeting the PI3K pathway.

#### STATEMENT OF TRANSLATIONAL RELEVANCE

PI3K $\alpha$  and its downstream signaling molecules are important therapeutic targets for molecularly defined subsets of cancer patients. In endometrial carcinomas, the occurrence of somatic mutations in the helical and kinase domains of p110 $\alpha$ , the catalytic subunit of PI3K $\alpha$ , has been well documented. Here we show that somatic, activating mutations in the ABD, ABD-linker region and C2 domains of p110 $\alpha$  are also very frequent in primary endometrial cancers. This finding identifies a novel subgroup of endometrial cancer patients, who might benefit clinically from targeted therapies directed against the PI3K-mediated pathway.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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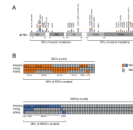
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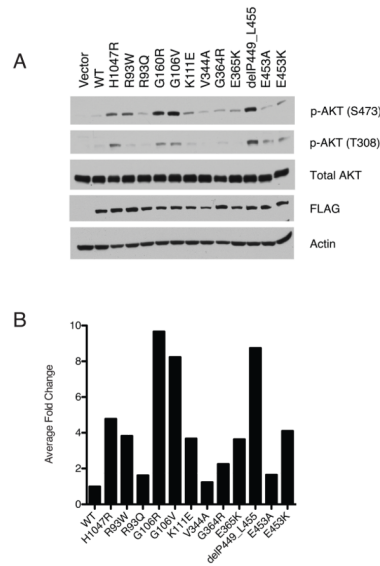
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**Figure 1. Distribution of somatic *PIK3CA* mutations in endometrial cancers and coexistence with *PTEN* and *KRAS* mutations**

(**A**) Localization of *PIK3CA* mutations in EECs (orange triangles) and NEECs (blue triangles), relative to functional domains of p110 $\alpha$ . Amino acid positions are indicated. (**B**) Patterns of *PIK3CA*, *PTEN*, and *KRAS* mutations among 42 EECs (top) and 66 NEECs (bottom). Each column represents an individual tumor. The percentage of mutated tumors is indicated. Cases with a mutation in exons 1–7 of *PIK3CA* are indicated by a central dot.



**Figure 2. A subset of amino terminal mutants of p110 $\alpha$  is associated with increased AKT phosphorylation on Ser473**

(A). Total protein from stably transfected, serum starved U2OS cells expressing vector, wild-type FLAG-p110 $\alpha$ , or mutant Flag-p110 $\alpha$  were analyzed by western blotting. Constructs expressing the known activating mutants, p110 $\alpha$ -H1047R and p110 $\alpha$ -E365K, served as positive controls for AKT phosphorylation (B) FLAG- p110 $\alpha$  and p-AKT<sup>Ser473</sup> bands were normalized to Actin. The ratio of normalized p-AKT<sup>Ser473</sup> to normalized FLAG-p110 $\alpha$  relative to wild-type p110 $\alpha$  is shown.

Table 1

Somatic *PIK3CA* mutations in EECs

Case	Tumor Histology	Tumor Stage	Tumor Grade	<i>PIK3CA</i> Mutation					Previously reported	
				Location	Nucleotide Change	Predicted Protein Change	Effect on Function †	In other cancers	In EEC or NEEC	
T87	Endometrioid	IB	II/III	Exon 1	c. G353A	G118D	-	Yes	No	
T88	Endometrioid	IB	I	Exon 1 Exon 12 Intron 6	c. G263A c. C2001A c. 1252-9 C>A	R88Q F667L Not determined	Activating <sup>28</sup>	Yes No -	Yes No -	
T89	Endometrioid	IIIA	I	Exon 1	c. A331G	K111E	Activating <sup>7</sup>	Yes	No	
T92	Endometrioid	IB	I	Exon 1 Exon 20	c. G278A c. C3074A	R93Q T1025N	- -	Yes Yes	No No	
T94	Endometrioid	IA	I	Exon 1 Exon 5	c. G317T c. G1090A	G106V G364R	Activating <sup>7</sup>	Yes No	No No	
T96	Endometrioid	IA	I	Exon 20	c. A3140G	H1047R	Activating <sup>10,31,32</sup>	Yes	Yes	
T97	Endometrioid	IIB	II	Exon 1 Exon 20	c. G263A c. A3062G	R88Q Y1021C	Activating <sup>28</sup>	Yes Yes	Yes Yes	
T99	Endometrioid	IA	I	Exon 1	c. del 325_327 GAA	del E109	-	Yes	No	
T101	Endometrioid	IB	I	Exon 7 Exon 20	c. T1258C c. A3140G	C420R H1047R	Activating <sup>32</sup> Activating <sup>10,31,32</sup>	Yes Yes	No Yes	
T102	Endometrioid	IB	II	Exon 7	c. del 1343_1363	del P449-L455	Activating <sup>7</sup>	No	No	
T103	Endometrioid	IB	II	Exon 9	c. G1624A	E542K	Activating <sup>32</sup>	Yes	Yes	
T115	Endometrioid	IB	I	Exon 20 Exon 20	c. A3118T c. A3140G	M1040L H1047R	- Activating <sup>10,31,32</sup>	No Yes	No Yes	
T116	Endometrioid	IA	II	Exon 1	c. C277G	R93W	Activating <sup>7</sup>	Yes	No	
T117	Endometrioid	IB	I	Exon 9	c. A1633G	E545K	Activating <sup>32</sup>	Yes	Yes	
T118	Endometrioid	IIA	I	Exon 20	c. A3140G	H1047R	Activating <sup>10,31,32</sup>	Yes	Yes	
T121	Endometrioid	IA	I	Exon 9	c. A1634G	E545G	Activating <sup>32</sup>	Yes	Yes	
T122	Endometrioid	IC	II	Exon 20	c. A3140G	H1047R	Activating <sup>10,31,32</sup>	Yes	Yes	
T123	Endometrioid	IB	I	Exon 9	c. C1636A	Q546K	Activating <sup>32</sup>	Yes	Yes	

Case	Tumor Histology	Tumor Stage	Tumor Grade	PIK3CA Mutation					Previously reported	
				Location	Nucleotide Change	Predicted Protein Change	Effect on Function <sup>†</sup>	In other cancers	In EEC or NEEC	
T131	Endometrioid	IIB	III	Exon 1	c. C277T	R93W	Activating <sup>‡</sup>	Yes	No	
T132	Endometrioid	IB	I	Exon 20	c. G3012T	M1004I	-	Yes	No	
T133	Endometrioid	IB	I	Exon 20	c. G3145C	G1049R	Activating <sup>28</sup>	Yes	Yes	
T134	Endometrioid	IC	I	Exon 9 Exon 20	c. G1633A c. A3127G	E545K M1043V	Activating <sup>32</sup> Activating <sup>32</sup>	Yes Yes	Yes Yes	

<sup>†</sup> Indicates the appropriate reference

<sup>‡</sup> Mutation shown to be activating in this study (Rudd et al)



**Table 2**

Somatic *PIK3CA* mutations in NEECs

Case	Tumor Histology	Tumor Stage	<i>PIK3CA</i> Mutation					
			Location	Nucleotide Change	Predicted Protein Change	Effect on Function†	Previously Reported	
							In other cancers	In EEC or NEEC
OM1323	Serous	I	Exon 1 Intron 3 Intron 8	c.G241A c.562+23 C>A c.1404+10 T>G	E81K Not determined Not determined	- - -	Yes - -	No - -
OM2009	Serous	I	Exon 9	c.A1634C	E545A	Activating <sup>32</sup>	Yes	Yes
T29	Serous	IB	Exon 9	c.G1624A	E542K	Activating <sup>32</sup>	Yes	Yes
T41	Serous	IIIA	Exon 1	del c.45-c.67 ins TCCAA	del L15_V22insHPI	-	No	No
T49	Serous	IIB	Exon 9	c.G1624C	E542Q	-	Yes	Yes
T53	Serous	IB	Exon 9	c.A1637C	Q546P	Activating <sup>32</sup>	Yes	No
T68	Serous	IIA	Exon 1 Exon 1	c.G263A c.G323A	R88Q R108H	Activating <sup>28</sup> Activating <sup>28</sup>	Yes Yes	Yes Yes
T69	Serous	IIIA	Exon 20	c.T3132A	N104K	-	Yes	Yes
T71	Serous	IB	Exon 5	c.G1093A	E365K	Activating <sup>28</sup>	Yes	Yes
T74	Serous	IB	Exon 20 Exon 1 Exon 1	c.C3139T c.G278A c.G333T	H1047Y R93Q K111N	Activating <sup>32</sup> - Activating <sup>32</sup>	Yes Yes Yes	Yes No No
T75	Serous	IIIA	Exon 20 Exon 20	c.A3172T c.A3207G	I1058F X1069_X1069insW KDN	- -	Yes Yes	Yes Yes
T76	Serous	IA	Exon 7 Intron 7	c.G1357A c.1404+3msT	E453K Not determined	Activating <sup>¶</sup> -	Yes -	No -
T78	Serous	IB	Exon 1	c.A331G	K111E	Activating <sup>¶</sup>	Yes	No
T79	Serous	IB	Exon 20	c.C3075A	T1025A	-	Yes	Yes
T80	Serous	IIIA	Exon 1	c.G263A	R88Q	Activating <sup>28</sup>	Yes	Yes
T81	Serous	IB	Exon 20	c.A3140G	H1047R	Activating <sup>10,31,32</sup>	Yes	Yes
T59	Clear cell	IIIA	Exon 4 Exon 1	c.T1031C c.G263A	V344A R88Q	- Activating <sup>28</sup>	Yes Yes	No Yes

Case	Tumor Histology	Tumor Stage	PIK3CA Mutation					Previously Reported	
			Location	Nucleotide Change	Predicted Protein Change	Effect on Function†	In other cancers	In EEC or NEEC	
T61	Clear cell	IC	Exon 7	c.A1358C	E453A	-	No	No	
T63	Clear cell	IIB	Exon 20 Exon 1	c.C3197T c.G278A	A1066V R93Q	-	Yes Yes	Yes No	
T77	Clear cell	IC	Exon 20	c.A3140G	H1047R	Activating <sup>†</sup> , <sup>‡</sup> , <sup>§</sup>	Yes	Yes	
T82	Clear cell	IVB	Exon 1	c.G316C	G106R	Activating <sup>‡</sup>	No	No	
T113	Clear cell	IB	Exon 3 Exon 5	c.C665T c.G1093A	A222V E365K	- Activating <sup>‡</sup>	No Yes	No Yes	

† Indicates the appropriate reference

‡ Mutation shown to be activating in this study (Rudd et al)