PIK3CA Cooperates with Other Phosphatidylinositol 3′-Kinase Pathway Mutations to Effect Oncogenic Transformation

Katsutoshi Oda,1,3 Jennifer Okada,1 Luika Timmerman,1 Pablo Rodriguez-Viciana,1 David Stokoe,2 Keiko Shoji,3 Yuji Taketani,3 Hiroyuki Kuramoto,3 Zachary A. Knight,2 Kevan M. Shokat,2 and Frank McCormick1

1Cancer Research Institute and 2Department of Cellular and Molecular Pharmacology, Howard Hughes Medical Institute, University of California at San Francisco, San Francisco, California; 3Department of Obstetrics and Gynecology, University of Tokyo, Tokyo, Japan; and 4Department of Clinical Cytology, Kitasato University Graduate School of Medical Sciences, Kanagawa, Japan

Abstract

Mutations in genes functioning in different pathways frequently occur together in the same cancer, whereas mutations in the same pathway tend to be mutually exclusive. However, the majority of colon, breast, and endometrial cancers that possess mutations in PIK3CA, the catalytic subunit p110α of phosphatidylinositol 3′-kinase (PI3K), also possess mutations or alterations in genes upstream of PI3K such as Ras, ERBB2/ERBB3, or PTEN. PIK3CA mutations occur almost exclusively in invasive tumors, whereas upstream mutations occur as frequently in early-stage and late-stage tumors, suggesting that PIK3CA mutation is a late-stage event that may augment earlier activation of the PI3K pathway. Consistent with this, we find that levels of p-AKT (Ser473) induced by mutant Ras or knockdown of PTEN were dramatically increased by addition of mutant PIK3CA. Soft agar assays revealed that anchorage-independent growth induced by mutant Ras was greatly increased in the presence of mutant PIK3CA. In breast, colon, and endometrial cancers in which the PI3K pathway is activated by a combination of mutant PIK3CA and alterations in Ras, ERBB2/3, or PTEN, signaling to downstream elements such as Akt was mediated exclusively by the p110α isoform, rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms. Our data therefore suggest that in tumors with co-occurring rather than a combination of different PI3K isoforms.

Introduction

The phosphatidylinositol 3′-kinases (PI3K) activate Akt and a wide range of downstream effectors to regulate multiple cellular activities, including cell proliferation, survival, and migration (1). Numerous activators of PI3K signaling are mutated or overexpressed in cancer, including receptor tyrosine kinases (EGFR mutation and ERBB2 overexpression; refs. 2, 3), Ras (4, 5), PTEN (6, 7), and PIK3CA, which encodes the catalytic subunit p110α of PI3K (8). Several types of alterations in this pathway are reported to be mutually exclusive [e.g., N-Ras mutations and PTEN mutations in melanoma (9) and loss of PTEN expression and PIK3CA mutations in breast cancer (10)]. However, it has become evident that some types of alterations in the PI3K pathway coexist in several types of tumors. We previously reported that PIK3CA mutations frequently coexist with PTEN mutations in endometrial cancer (11). Saal and colleagues (10) reported that ERBB2 overexpression associates with PIK3CA mutations in breast cancer. In addition, Parsons and colleagues (12) showed that 27 of 37 colorectal cancers with PIK3CA mutations also possess k-ras mutations. As summarized in Supplementary Table S1, K-ras mutations in the colorectum, ERBB2 overexpression in the breast, and PTEN mutations in the endometrium are as commonly observed in premalignant (noninvasive) tumors (38–75%, 34–50%, and 18–48%, respectively) as in malignant tumors (43–65%, 16–29%, and 26–57%, respectively; refs. 4, 13–19). On the other hand, in spite of the common prevalence in many malignant tumors, PIK3CA mutations are rare in noninvasive disease of these tissues; the ratio is 6% in the colorectum (14), 0% in the breast (20), and 7% in the endometrium (19). Moreover, the coexistence of PTEN and PIK3CA mutations was not detected in any noninvasive disease of the endometrium (19). Thus, K-Ras, ERBB2, and PTEN alterations readily occur in noninvasive tumors, whereas PIK3CA mutations occur later, during or after invasion.

Class I PI3Ks are activated by receptor tyrosine kinases and comprise one of several signaling activities induced by activated Ras. Their activity is required for Ras contribution to cellular transformation (21) and is produced by four isoforms of the class I catalytic subunit (p110α, p110β, and p110δ for class IA, and p110γ for class IB; ref. 22). Although p110α is the only subunit in which activating mutations have been reported, the other three isoforms also have oncogenic potential (23). Indeed, p110β is more strongly activated than p110α in a PTEN-null prostate cancer cell line (PC-3; ref. 24). Thus, the activity of non-PIK3CA (non-p110α) isoforms, induced by upstream signaling events, might be functioning in early stages of tumor formation and augmented by activating mutations in PIK3CA to potentiate total PI3K signaling during malignant progression. Alternatively, the majority of PI3K oncogenic activity might always occur via PIK3CA, with a mutational event in this gene enforcing signaling duration or intensity requisite for later transformation events.

In this study, we first identify patterns of naturally occurring coexistent alterations in the PI3K pathway in endometrial and breast cancer. Second, we show that PI3K signaling is weakly activated by various upstream PI3K pathway alterations alone and is augmented when mutant p110α is coexpressed. Third, we show that mutant p110α, in combination with mutant Ras, contributes to efficient malignant transformation of immortalized human mammary epithelial cells (HMEC). Lastly, we show that the p110α isoform is

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Katsutoshi Oda, Department of Obstetrics and Gynecology, University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan. Phone: 81-3-5800-8687; Fax: 81-3-3816-2017; E-mail: katsutoshi-tky@umin.ac.jp.

©2008 American Association for Cancer Research.
most responsible for PI3K pathway activation in tumors with coexistent PIK3CA mutations and other PI3K activating alterations.

**Materials and Methods**

**Cell lines and retroviral infection.** The culture condition of cell lines is described in Supplementary data. HMLE (HMECs with LT and hTERT) cells and HMLE cells with pBabe-zeo-H-Ras (G12V) and pBabe-puro-H-Ras (G12V) were a kind gift from Dr. Robert A. Weinberg. pBabe-zeo-H-Ras (G12V) clone expressed H-Ras at a lower level than the pBabe-puro-H-Ras (G12V) clone (7.2- versus 12-fold in comparison with endogenous level; ref. 25).

To create amphotropic retroviruses, Phoenix cells were transfected with retroviral vectors using Lipofectamine 2000 (Invitrogen): pFB-neo-p110a [wild-type (WT) and mutant], pFB-neo-GFP (green fluorescent protein), and pLXSP3-puro-K-Ras (G12V). The resulting supernatants were used to infect PAE, U2OS, and HMLE cells. Drug selection was used to purify cell populations after infections. Cells were selected in neomycin (500–1,500 μg/mL, 7d) and puromycin (0.5–1.5 μg/mL, 3 d). Retroviral vectors carrying only drug resistance genes were used as controls. PTEN knockdown was achieved by infection of retrovirus expressing PTEN-specific short hairpin RNA (shRNA; Open Biosystems) and drug selection in puromycin (0.5–1.5 μg/mL, 3 d). We successfully introduced mutant p110a (H1047R) and GFP with neomycin selection at 1,500 μg/mL in HMECs, although neomycin had been used at 200 μg/mL for LT selection (most of the cells were GFP positive in the neo-GFP clone; data not shown).

**Endometrial tumor samples and genomic DNA.** The clinical characteristics and genomic DNA extraction of 66 endometrial tumors were described previously (11, 26). All of the patients provided informed consent for the research use of their samples, and the collection and use of tissues for this study were approved by the appropriate institutional ethics committees.

**Statistical analysis.** The association of variables was evaluated by the Fisher’s exact test. \( P < 0.05 \) was considered to be significant in all the tests.

**Quantification of protein and mRNA levels.** As semi-quantitative measurement of PTEN protein levels, p-ERBB2 (Tyr\(^{1248}\)), p-ERBB3 (Tyr\(^{1289}\)), total ERBB2, and total ERBB3 in breast cancer cell lines was determined from TIFF files of Western blots using Scion Image software.\(^5\) Protein levels across the Westerns were normalized using the common controls within each blot. The PTEN expression is considered “reduced” when the expression level is <25% compared with the average from positive cell lines. ERBB2 and ERBB3 are considered overexpressed when the mRNA

---

**Table 1. Coexistent mutations of PIK3CA and K-Ras and/or PTEN in endometrial cell lines**

<table>
<thead>
<tr>
<th></th>
<th>K-Ras</th>
<th>PTEN</th>
<th>PIK3CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutated codon</td>
<td>Mutated exon (type*)</td>
<td>Mutated codon</td>
<td></td>
</tr>
<tr>
<td>HEC-1B</td>
<td>G12D</td>
<td>WT</td>
<td>G1049R</td>
</tr>
<tr>
<td>HHUA</td>
<td>G12V</td>
<td>5 (F), 8 (F)</td>
<td>R88Q</td>
</tr>
<tr>
<td>HEC-6</td>
<td>WT</td>
<td>4 (F), 8 (F)</td>
<td>R108H</td>
</tr>
<tr>
<td>HEC-59</td>
<td>WT</td>
<td>2 (P), 7 (N), 7(P), 7(F)</td>
<td>R38C</td>
</tr>
<tr>
<td>HEC-88</td>
<td>WT</td>
<td>5 (P), 6 (P), 8 (P), 8 (P)</td>
<td>E365K</td>
</tr>
<tr>
<td>HEC-116</td>
<td>WT</td>
<td>6 (P), 7 (N)</td>
<td>R88Q</td>
</tr>
<tr>
<td>HEC-108</td>
<td>WT</td>
<td>1 (F), 8(F)</td>
<td>WT</td>
</tr>
<tr>
<td>HEC-151</td>
<td>WT</td>
<td>2 (P), 4(F)</td>
<td>WT</td>
</tr>
<tr>
<td>AN3CA</td>
<td>WT</td>
<td>5 (N)</td>
<td>WT</td>
</tr>
<tr>
<td>Ishikawa 3-H-12</td>
<td>WT</td>
<td>8 (F), 8 (F)</td>
<td>WT</td>
</tr>
<tr>
<td>RL-95-2</td>
<td>WT</td>
<td>8 (F), 8 (F)</td>
<td>WT</td>
</tr>
<tr>
<td>Hec-50</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>KLE</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

**B. Relationship among the genetic lesions**

<table>
<thead>
<tr>
<th>PTEN</th>
<th>Mutant</th>
<th>PIK3CA mutation (%)</th>
<th>( P = 0.061 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>22/47 (47)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8/32 (25)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>K-ras</th>
<th>Mutant</th>
<th>PIK3CA mutation (%)</th>
<th>( P = 0.075 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>9/15 (60)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>21/64 (33)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PTEN and/or K-ras</th>
<th>Mutant</th>
<th>PIK3CA mutation (%)</th>
<th>( P = 0.084 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>24/53 (45)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6/26 (23)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PTEN</th>
<th>Mutant</th>
<th>K-ras mutation (%)</th>
<th>( P = 1.00 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>9/47 (19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6/32 (19)</td>
<td></td>
</tr>
</tbody>
</table>

\*F, frameshift mutation; P, point mutation; N, nonsense mutation.

---

level in expression array hybridization is more than 4- and 15-fold, compared with that of the nontumorigenic cell line MCF10A, respectively. “Intermediate” ERBB3 expression corresponds to a 10- to 15-fold increase from that in MCF10A.

Small interfering RNA transfection. Small interfering RNA (siRNA) was used to inhibit the expression of ERBB2, ERBB3, p110α, or p110β. The targeted sequences of ERBB2, p110α, and p110β siRNAs are 5'-AAGGTGCTTGGATCTGGCGCT-3', 5'-AAGGAGCCCAAGAATGCACAA-3', and 5'-AAAGGGAGCGAGTGCCTTTTA-3', respectively. siRNA to ERBB3 (siGenome) was purchased from Dharmacon RNA Technologies. Cells were seeded at 2.0 × 10^5 per six-well plate 24 h before transfection and transfected with 60 to 120 nmol/L siRNA duplexes using Lipofectamine 2000 (Invitrogen). Cells were collected 72 h after transfection and analyzed by immunoblotting. Suppression of p110α and p110β by siRNA was confirmed by real-time PCR.

Cluster analysis. Unsupervised Cluster analysis was done on an Affymetrix U133A–based breast tumor cell line data set (27) using Cluster and Treeview software. Average linkage clustering results using the 15,000 array elements, which exhibit the most variance across the data set, are reported. Red squares represent high and green squares represent low relative expression levels of ERBB2 and ERBB3.

Immunoblotting. Cells were lysed as described previously (11). Antibodies to PTEN, phospho-PTEN (Ser 380), total Akt, phospho-Akt (Ser 473), total p110α, total GSK3β, phospho-GSK3β (Ser 9), total S6,

Figure 1. Coexistent PIK3CA mutation and ERBB2 and/or ERBB3 overexpression in breast cancer cell lines. A, alterations of ERBB2, ERBB3, PTEN, and PIK3CA in a panel of breast cancer cell lines, grouped by unsupervised cluster analysis. Relative ERBB2 and ERBB3 expression levels are indicated as output from the cluster analysis (red, overexpression; green, underexpression). Protein expression levels of PTEN and phosphorylation of Akt (Ser 473) and p-ERBB2 (Tyr 1248) were assessed by Western blotting. PIK3CA mutant cell lines (marked with green asterisk) were derived from the Sanger Center COSMIC database. The nontumorigenic cell line MCF10A is shown with a box. B, relationship among the alterations in A. PIK3CA mutations correlate with ERBB2 and/or ERBB3 overexpression.
phospho-S6 (Ser\(^{235/236}\)), total FoxO1, phospho-FoxO1 (Thr\(^{24}\)), phospho-FoxO3a (Thr\(^{32}\)) (Cell Signaling Technology), \(\beta\)-actin, FLAG tag (Sigma-Aldrich), total K-Ras, total ERBB2, total ERBB3 (Santa Cruz Biotechnology), and phospho-ERBB2 (Tyr\(^{1248}\)) (LabVision Corp.) were used for immunoblotting as recommended by the manufacturer and were detected with enhanced chemiluminescence Western blot detection kit (Amersham Biosciences) or Immobilon Western detection reagents (Millipore Biosciences).

**Soft agar assays.** PAE, U2OS, and HMLE cells were seeded at \(1 \times 10^3\) or \(4 \times 10^3\) per well in six-well dishes with a bottom layer of 0.6% agar in DMEM and a top layer of 0.35% agar in DMEM (for PAE and U2OS) or mammary epithelium growth medium (for HMLEs). Fresh medium (0.5 mL; DMEM for PAE and U2OS, mammary epithelium growth medium for HMLEs) was added after 1.5 wk. Colonies (>0.2 mm in diameter) were counted after 2 to 3 wk. At least two independent assays were done in triplicate.

**Results**

**PIK3CA mutations coexist with K-ras and PTEN mutations in endometrial carcinomas.** We screened 79 endometrial carcinomas (66 clinical specimens and 13 cell lines) for mutations of K-ras, PIK3CA, and PTEN. The mutational status of 13 endometrial cancer cell lines is shown in Table 1A. Overall, the mutation frequency in 79 endometrial cancers is 19% for K-ras, 57% for PTEN, and 37% for PIK3CA. Tumors with K-ras mutation exhibited a tendency to carry PIK3CA mutation more frequently (9 of 15, 60%) than tumors without K-ras mutation (21 of 64, 33%), although statistical significance was not reached (\(P = 0.075\), Fisher’s exact test). We did not find any association between PTEN mutations and K-ras mutations (9 of 47 versus 6 of 32, \(P = 1.0\); Table 1B), suggesting that mutation of these two genes occurs independently. As shown in Table 1B, 24 of 30 (80%) tumors with PIK3CA mutations possess K-ras and/or PTEN mutations.

**PIK3CA mutations are associated with ERBB2 and/or ERBB3 overexpression in breast cancer cell lines.** We screened a panel of 47 breast cancer cell lines for activators of PI3K signaling. The mutational status of PIK3CA was determined by examination of the COSMIC database (Sanger Center).\(^6\) ERBB2, ERBB3, PTEN, and p-Akt (Ser\(^{473}\)) levels were assessed by expression array hybridization and/or Western blotting (Fig. 1; summarized in Supplementary Table S2). We focused on ERBB2 and ERBB3 expression levels in breast cancer because (a) the association between PIK3CA mutations and ERBB2 overexpression is still controversial (28); (b) the ERBB2/ERBB3 heterodimer functions as an oncogenic unit (29); and (c) ERBB3 overexpression in breast cancer is also common (30). In addition, ERBB3 contains six

\(^6\)www.sanger.ac.uk/genetics/CGP/cosmic/
docking and activation sites for PI3K, and p-ERBB3 is shown to activate the PI3K pathway (31). Micro-RNAs miR-125a and miR-125b, which suppress both ERBB2 and ERBB3 expression, are down-regulated in breast cancers (32), suggesting that ERBB2/ERBB3 signaling is important for breast tumorigenesis. Relative to expression levels found in the nontumorigenic cell line MCF10A, we found ERBB2 and/or ERBB3 overexpression in 19 of 47 (40%) cell lines; 10 with ERBB2 overexpression and 13 with ERBB3 overexpression, including four (BT474, HCC202, UACC-812, and SUM225CEM) with overexpression of both molecules. PIK3CA mutations were positive in 13 (28%) cell lines (Fig. 1A; Supplementary Table S2). Nine of 13 (69%) with PIK3CA mutations harbor ERBB2 and/or ERBB3 overexpression ($P = 0.020$, Fisher’s exact test; Fig. 1B). On the other hand, reduced PTEN expression (11 of 47, 23%) was less common in cells with PIK3CA mutations (1 of 13, 8%), compared with those without PIK3CA mutations (10 of 34, 29%; $P = 0.15$). In addition, reduced PTEN expression was inversely correlated with ERBB2 and/or ERBB3 overexpression ($P = 0.032$). Elevated phosphorylation of Akt (20 of 47, 43%) was significantly associated with PIK3CA mutations and/or reduced PTEN expression ($P = 0.019$). In contrast to endometrial carcinomas, loss of PTEN expression is reported to be very rare in noninvasive breast cancer (ductal carcinoma in situ) and more common in advanced stage tumors (33). Thus, loss of PTEN may be a late event in breast cancer. Semiquantitative analysis of phospho-ERBB2 (Tyr1248) and phospho-ERBB3 (Tyr1289) levels (27) showed that overexpression of each molecule was well associated with the level of its phosphorylation and p-Akt (Fig. 1A; Supplementary Table S3). Six of nine PIK3CA mutated tumors showed high p-ERBB2 and/or p-ERBB3 levels (Supplementary Table S3). Our data indicate that PIK3CA mutations frequently coexist with active ERBB2/ERBB3 signaling in breast carcinomas.

**Mutant p110α is more potent in phosphorylation of Akt than mutant Ras or reduced PTEN expression.** According to our previous report that multiple alterations in the PI3K pathway could further activate PI3K signaling in endometrial cell lines (11), we

---

**Figure 3.** Anchorage-independent growth is induced by the combination of mutant Ras and mutant p110α in immortalized HMEC cells. A, soft agar assay in HMECs expressing mutant H-Ras and/or mutant p110α. One thousand cells were seeded and cultured for 14 d and stained with neutral red. Columns, mean number of soft agar colonies from three experiments; bars, SD. B, soft agar assay in HMECs expressing mutant K-Ras and/or mutant p110α. Four thousand cells were seeded and treated as described in A. Columns, mean number of soft agar colonies from three experiments; bars, SD.
hypothesized that the presence of the upstream PI3K activation might cooperate with mutant p110α to increase PI3K activity, and that the presence of mutant p110α might be important for oncogenic conversion or progression. Therefore, we introduced mutant K-(or N-) Ras (G12V), mutant p110α (H1047R), shRNAs that targeted PTEN, and combinations of these alleles into PAE (porcine aortic endothelial) and U2OS osteosarcoma cells by retrovirus-mediated gene transfer. Both PAE cells and U2OS cells do not harbor any mutations in Ras, PTEN, and PIK3CA, resulting in very low basal p-Akt levels. Either PTEN knockdown or mutant Ras expression effected the p-Akt at a much lower level than did mutant p110α alone in both cells (Fig. 2A and B). The phosphorylation level of FoxO1, FoxO3a, and GSK3β was enhanced by either mutant K-Ras or PTEN silencing when combined with mutant p110α (Fig. 2A). Transient introduction of mutant Ras increased the p-Akt level in U2OS cells stably expressing mutant p110α (Fig. 2C). In summary, either mutant Ras or PTEN silencing alone has limited effect on the activation of the PI3K pathway, but the effect is much more drastic when combined with mutant p110α.

Anchorage-independent growth is induced by mutant p110α in PAE and U2OS cells. We tested whether mutant p110α might induce oncogenic behavior (anchorage-independent growth) in the PAE and U2OS cell lines because both PAE (untransformed) and U2OS cells are unable to grow without attachment to a substratum (34). Stable expression of mutant p110α (H1047R) resulted in growth of colonies in soft agar in both cells (Supplementary Fig. S1A and B), suggesting that the higher PI3K activity via mutant p110α is involved in anchorage-independent growth in these cells.

Mutant Ras and p110α efficiently induced oncogenic transformation in immortalized HMEC cells. Because alterations of Ras or ERBB2 are observed in noninvasive tumors of the colorectum, breast, and endometrium, we hypothesized that activation of multiple Ras effectors is useful for tumor initiation and that higher PI3K activation via additional mutations in p110α...
is helpful for malignant progression of noninvasive tumors. In addition, because PIK3CA mutations without other alterations are rare in these tumor types, mutant p110α alone might be insufficient for oncogenic transformation. Mutant H-Ras (G12V) transforms immortalized HMECs only when it is highly overexpressed (25); we assumed that additional mutant p110α might increase transformation efficiency in cells expressing lower levels of mutant Ras. To test this hypothesis, we obtained HMECs previously immortalized with large T-antigen and TERT (HMLE), with and without various alleles of activated H-Ras (25), and introduced activating alleles of p110α, K-Ras, or PTEN-silencing shRNAs in various combinations to determine their relative transformation potential (summarized in Supplementary Table S4).

Akt was more potently activated by introduction of mutant p110α than by mutant (H- or K-) Ras or PTEN shRNA (Supplementary Fig. S2A and B). The transformation potential of these stable cell lines was then assessed by soft agar assay. Mutant p110α (H1047R) alone failed to cause anchorage-independent growth in the parental HMLE (Fig. 3A). In addition, mutant p110α plus partial reduction of PTEN expression by shRNA did not cause anchorage-independent growth. However, in HMLE sublines, which constitutively express zeo-H-Ras (G12V) or puro-H-Ras (G12V), addition of mutant p110α (H1047R) doubled the colony number relative to control GFP (Fig. 3A). Unlike mutant H-Ras, introduction of mutant K-Ras (G12V) alone did not induce anchorage-independent growth; however, the
combination of mutant p110α (H1047R) and mutant K-Ras enabled cells to grow in soft agar (Fig. 3B).

**p110α is the predominant form of PI3K activity in breast cancer cells with coexistent mutant p110α and overexpression of ERBB2 and/or ERBB3.** To determine which p110 isoforms were activated by upstream PI3K pathway activating alterations in cells with PIK3CA mutation, we examined the effect of p110 isoform-selective inhibitors in five breast cancer cell lines with coexistent PIK3CA mutations and ERBB2 and/or ERBB3 overexpression (SUM185PE, MDA-MB361, and T47D: ERBB3 overexpression; HCC202 and BT474: both ERBB2 and ERBB3 overexpression). Four types of p110 inhibitors were tested on each cell line at 2 or 5 μmol/L for 1 to 6 h. These include the selective p110δ inhibitor TGX-286; the selective p110δ inhibitor PIK39; PIK85, which selectively inhibits p110α, p110γ, and p110δ; and the pan-selective p110 inhibitor PI-103. The structure of these four compounds is available from ref. 35 and Supplementary Fig. S3. The IC_{50} values of the four compounds were listed in Supplementary Table S5. Both PIK39 (p110δ inhibitor) and TGX-286 (p110δ inhibitor) failed to suppress PI3K signaling as assessed by phosphorylation of Akt (Ser473) and GSK-3β (Ser21), whereas PIK85 (p110α, p110γ, and p110δ inhibitor) drastically suppressed phosphorylation of these molecules to a level similar to that of PI-103, wortmannin, and Ly294002 (pan-selective agents) in all these breast cancer cell lines (Fig. 4A and B, and data not shown). The p110γ hybridization signal is low to undetectable in the analysis of Affymetrix gene expression profiling data (27) in breast cancer cell lines, indicating that little, if any, of this mRNA is expressed in the breast, whereas p110α, p110γ, and p110δ are expressed at comparable and more robust levels. These data suggest that breast cancer cells line with mutant p110α and overexpression of ERBB2 and/or ERBB3 are dependent on p110α activity, rather than other isoforms, for activation of the PI3K pathway. Transfection with p110α-specific siRNA, but not with p110β-specific siRNA, suppresses Akt phosphorylation in BT474 cells (Fig. 4C). The p-Akt level was also decreased in SUM185PE cells by p110α-specific siRNA (Fig. 4C). These data also suggest that p110α is the major activated form in these cells.

To identify the contribution of ERBB2 and/or ERBB3 overexpression on the activity of the PI3K pathway, we used siRNA knockdown to reduce the expression of ERBB2 or ERBB3 in BT474. Attenuating the expression of these proteins partially reduces the level of p-Akt (Fig. 4D). ERBB3 suppression reduced the p-Akt level in SUM185PE cells (Fig. 4D). In addition, serum starvation decreased the p-Akt level in all five cell lines (data not shown). These data indicate that activation of PI3K signaling depends not only on mutant p110α but also on ERBB2/ERBB3 overexpression and/or serum-derived growth factors.

**p110α is predominantly active in colorectal cancer cells with double mutations of p110α and Ras.** The pattern of coexistent alterations in the PI3K pathway in colorectal cancer is double mutations of PIK3CA and K-ras. To determine whether PI3K activity with these coexistent mutations also relies primarily on p110α, we tested the p110 isoform-selective inhibitors in two colorectal cancer cell lines with these double mutations (DLD1 and HCT116). The phosphorylation of Akt was sensitive to PIK85 and PI-103 but resistant to TGX-286 and PIK39 (Supplementary Fig. 4A and B) in these two cells. These data suggest that double mutations of K-ras and PIK3CA predominantly activate the p110α isoform in colorectal cancer.

**p110α is predominantly active in endometrial cancer cells with double mutations of PIK3CA and PTEN.** Next, we analyzed five endometrial cancer cell lines (HHUA, HEC-59, HEC-6, HEC-116, and HEC-88) with double mutations of PIK3CA and PTEN (Table 1A). PIK85 and PI-103, but neither TGX-286 nor PIK39, drastically decreased the level of p-Akt in all five cell lines (Fig. 5A–C; Supplementary Fig. S5A and B). In addition, PIK85, PI-103, and wortmannin suppressed the phosphorylation of Akt substrates such as p-GSK3β (Ser9), p-FoxO1 (Thr32), and p-S6 (Ser235/236; Fig. 5A–C; Supplementary Fig. S5A and B). Reduction of p110α by siRNA also suppressed Akt phosphorylation in HeC-6 cells, whereas reduction of p110γ by siRNA did not (Supplementary Fig. S5C). These data indicate that p110α is constitutively activated in endometrial tumor cells with double mutations of PTEN / PIK3CA.

**Discussion**

These results highlight the important role of PIK3CA mutations during oncogenic transformation in multiple epithelial tumor types. In this study, we addressed the meaning of multiple alterations in the PI3K pathway by analyzing (a) the patterns of coexistent alterations, (b) the efficiency of PI3K activation by single alterations, (c) the oncogenic transformation by coexistent alterations, and (d) the p110 isoform selectivity. These issues are discussed in more detail below.

**Coexistent PIK3CA mutations and other alterations in endometrial, breast, and colorectal cancers.** Endometrial, breast, and colorectal cancers are three major tumor types that show a high frequency of PIK3CA mutations (8, 11, 36). Curiously, the partners of PIK3CA mutations differ between these tissues: PTEN mutations in endometrium, ERBB2 overexpression in breast, and K-ras mutations in colorectum were reported to commonly coexist with PIK3CA mutations (10–12). In this study, we further identified double mutations of K-ras and PIK3CA in endometrial cancer and the coexistence of PIK3CA mutation and ERBB2/ERBB3 signaling in breast cancer cell lines. Various patterns of coexistent alterations of PIK3CA and the genes upstream of PI3K suggest that the role of mutant PIK3CA might be different from the other alterations. Ras and ERBB2/ERBB3 are involved in various signaling pathways, whereas PTEN also has PI3K-independent functions. Therefore, the co-occurring alterations in the PI3K pathway may have two major roles: augmentation of the PI3K pathway and activation of other pathways.

Although PIK3CA mutations in six endometrial cell lines are outside the three well-characterized mutation hotspots (E542, E545, and H1047; refs. 22, 37), all five p110α mutants (R38C, R88Q, R108H, E365K, and G1049R) significantly increased the phosphorylation levels of Akt, GSK3β, FoxO1, FoxO3a, and S6 compared with WT p110α in the U2OS sublines (Supplementary Fig. S6). These data indicate that these rare p110α mutations cause a gain of PI3K function.

**Enhanced activation of the PI3K pathway by additional mutant p110α.** Combined PIK3CA mutation and other alterations in the Ras-PI3K pathway (PTEN and/or K-ras mutations in endometrium, ERBB2 and/or ERBB3 overexpression in breast, and K-ras mutation in colorectum) have two common features: First, these other alterations already exist in noninvasive tumors, whereas PIK3CA mutation is rarely observed in noninvasive tumors. Second, the combined ratio (≥40%) of other alterations is higher than that of PIK3CA mutations (20–40%) in these cancers (Table 1B; Supplementary Table S1). These data suggest that activation of the PI3K pathway via any WT p110α isoform is not
sufficiently high in tumors with single alterations, although those tumors occasionally progress to invasive tumors without an additional PIK3CA mutation (possibly via other unknown alterations or compensation from other pathways). In our experiments, sole expression of mutant Ras or knocking down PTEN was less effective than mutant p110α, and addition of mutant p110α dramatically increased p-Akt levels in PAE, U2OS, and HMLE cells. Samuels and colleagues (38) established isogenic HCT116 and DLD1 colorectal cell lines in which either the WT or mutant alleles of PIK3CA were disrupted. Because both HCT116 and DLD1 possess mutant K-Ras (G13D), their analysis compared mutant K-Ras alone versus double mutants of K-Ras and PIK3CA. The PIK3CA mutant clones showed higher levels of p-Akt and p-FoxO1/p-FoxO3α compared with PIK3CA WT clones in both cell lines. Their data implied that mutant Ras alone is not sufficient for full activation of the PI3K pathway and that addition of the endogenous level of mutant p110α enhances the pathway, including some downstream effectors such as FoxO1/FoxO3α.

**Efficient oncogenic transformation by combined mutant Ras and mutant p110α.** Oncogenic transformation by mutant p110α was reported in chicken embryo fibroblasts in vitro and in vivo (37, 39). In addition, we found anchorage-independent growth by mutant p110α in PAE and U2OS cells (Supplementary Fig. S1A and B). However, it has not been clear whether mutant p110α alone is sufficient for malignant transformation in immortalized human epithelial cells. In HMLEs, mutant p110α failed to induce anchorage-independent growth (Fig. 3A), suggesting that activation of the PI3K pathway alone is insufficient for transformation. This is compatible with the observation that mutant p110α frequently coexists with other alterations in the Ras-PI3K pathway. Transformation by mutant Ras is well analyzed in various types of immortalized human epithelial cells (25, 40–42). However, low levels of mutant H-Ras alone failed to confer a full malignant phenotype (Fig. 3A; refs. 25, 40) or required another input in the PI3K pathway [constitutively active form of Akt (41) or SV40 small t antigen (42)]. In addition, mutant K-Ras did not induce anchorage-independent growth in HMLE (Fig. 3B). These observations suggest that endogenous levels of mutant Ras alone are insufficient for full transformation in immortalized human epithelial cells. However, mutant p110α significantly increased the number of colonies in soft agar when combined with mutant H-Ras or K-Ras. In our experiments, mutant Ras itself activated the PI3K pathway at a weaker level, but additional mutant p110α clearly enhanced the level of p-Akt, suggesting that the activity of the PI3K pathway was more strengthened. Our data indicate that additional mutant p110α plays a significant role in tumorigenicity and suggest that the enhanced PI3K activity might be responsible for the oncogenic activity.

The role of PTEN alterations in tumorigenesis is still unclear and may be tissue type specific. PTEN knockdown was incomplete in HMLE cells in our soft agar assay, although p-Akt was enhanced. That might be why the transformation efficiency might not be affected by PTEN shRNA. In addition, reduced PTEN expression in breast cancer is rare in noninvasive tumors and is instead associated with tumor invasion (33). However, PTEN mutations are common in atypical hyperplasia of the endometrium, as well as K-ras mutations (19, 43), and also frequent in ovarian endometriosis (44). In mouse models, either mutant K-Ras or conditional PTEN deletion within the ovarian surface gives rise to preneoplastic ovarian (endometriosis like) lesions and leads to invasive endometrioid ovarian cancer when combined (45). These observations indicate that PTEN alterations may have a similar effect with mutant K-Ras in tumor initiation and cooperate with mutant p110α in tumorigenesis of the endometrium and the ovary. Another possibility is that PI3K-independent PTEN functions (i.e., phosphorylation-independent function) may be important in the tumorigenesis. Therefore, each mutation of K-ras, PTEN, and PIK3CA may have different meanings in endometrial tumorigenesis.

The model of tumorigenesis in colorectal, breast, and endometrial tumors with coexistent PIK3CA and other alterations is summarized in Fig. 5D. Mutant Ras (or ERBB2 overexpression, etc.) is important for tumor initiation, which might be achieved through multiple Ras effector pathways, including the mitogen-activated protein kinase, Ral guanine nucleotide exchange factor, and PI3K pathways (21, 46, 47). However, the PI3K activation is not saturated in these noninvasive tumors. Once PIK3CA is mutated during the latent phase, the PI3K pathway could be activated to a greater level, which may result in efficient malignant transformation of noninvasive tumors. Without PIK3CA mutations, the noninvasive tumors may need another input (possibly PI3K pathway independent) to become transformed or they may need a longer latent phase. It is still unclear whether the augmented PI3K pathway activation alone is responsible for the transformation efficiency in PIK3CA mutated tumors. Mutant p110α may have any functions of cross talk with the other pathways, which are already activated by the earlier alterations. In addition, the magnitude of PI3K pathway activation is not always linked to the transformation efficiency. In PAE cells, mutant K-Ras induced more colony numbers than mutant p110α in soft agar assay (data not shown), suggesting that the requirement for PI3K pathway enhancement depends on the cellular context.

**Predominant p110α activation in tumors with coexistent mutant p110α and other PI3K-activating alteration(s).** Our observations with p110 isoform–selective inhibitors highlighted the importance of p110α activity in tumors with mutant p110α and other alterations. We used PIK-85 to inhibit p110α, p110γ, and p110δ. p110γ expression is suggested to be exclusively restricted to hematopoietic cells (48), and the expression in cancer cell lines was also at low to undetectable levels. PIK39 was useful to exclude p110δ activation, as it is a highly selective p110δ inhibitor. We had assumed that the higher PI3K activation seen with coexistent mutations in the PI3K pathway might be due to the activation of multiple p110 isoforms. In fact, we observed that several cancer cell lines with PTEN alterations alone (without PIK3CA mutations) in breast and endometrium showed some sensitivity to TGX-286 or PIK39 (data not shown), suggesting that loss of PTEN function is not always associated with activation of p110α. One possible explanation is that other alterations without a PIK3CA mutation may activate the PI3K pathway through multiple (or non-α) p110 isoform(s), and that isoform dependency might be switched to p110α once p110α is mutated. To clarify the mechanism of p110α isoform selectivity, cells from invasive and noninvasive tumors that do not harbor PIK3CA mutations but have alterations in other PI3K pathway elements might be studied to address this question.

Predominant p110α activation in PIK3CA mutant tumors indicates that p110α-selective inhibition might be a good therapeutic target in tumors with mutant p110α. Trastuzumab (Herceptin) is a well-established targeted therapy in breast cancer (49), but our data suggest that trastuzumab might not be sufficient to suppress the PI3K pathway in tumors with double alterations of ERBB2 and PIK3CA. p110α isoform–selective inhibition may be less toxic than pan-selective PI3K inhibition and may be more effective than trastuzumab in tumors with mutant p110α.
Conclusion

We confirmed that PIK3CA mutations frequently coexist with other alterations in the PI3K pathway in endometrium, breast, and colorectum. All cell lines with coexistent mutant p110α and other alterations showed p110α dependency and enhanced activity of the PI3K signaling pathway. Mutant p110α cooperates with mutant Ras to efficiently transform HMECs. Taken together, the high activity of p110α by a coexistent PIK3CA mutation with other alterations can be a critical step for malignant transformation.

Disclosure of Potential Conflicts of Interest

K. Oda: commercial research grant, Daiichi-Sankyo Pharmaceutical Co. Ltd. F. McCormick: commercial research grant and consultant, Daiichi-Sankyo and data.

References

1. Stokoe D, Stephens LR, Copeland T, et al. Dual role of phosphorylated-3,4,5-trisphosphate in the activa-
2. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underw
7. Kong D, Chen K, A. Zou TT, et al. PTEN1 is frequently mutated in primary endometrial carcinomas. Nat
10. Sual JL, Holm K, Maurer M, et al. PIK3CA mutations correlate with receptors, node metastasis, and
11. Oda K, Stokoe D, Takeuchi Y, et al. High frequency of coexistent mutations of PIK3CA and PTEN genes in
436:792.
13. Burmer GC, Loeb LA. Mutations in the KRAS2 oncoprotein during progressive stages of human colon
14. Kann I, Han J, Ahquist D, et al. Improved marker concentration for detection of de novo genetic variation and
15. Somerville JE, Clarke LA, Biggart JD. c-erbB-2 overexpression/amplification in the progression of ductal
16. Latta EK, Tjian S, Parkes BK, et al. The role of HER2/
neu overexpression/amplification in the progression of ductal carcinoma in situ to invasive breast carcinoma.
17. Park K, Han S, Kim HJ, et al. HER2 status in pure ductal carcinoma in situ and in the intraductal and
21. Lim KH, Counter CM. Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT
24. Schwarzer B, Tondera D, Arnold W, et al. REDD1 integrates hypoxia-mediated survival signaling down-
26. Minaguchi T, Yoshikawa H, Oda K, et al. PTEN mutation located only outside exons 5, 6, and 7 is an
independent predictor of favorable survival in endome-
28. Buttitta F, Felicioni L, Barassi F, et al. PIK3CA mutations and histological type in breast carcinoma:
requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A 2003;100:8953–8.
1998;78:1385–90.
with hormone receptors, node metastasis, and Pten in the development of mouse models of
32. Zhao JJ, Gjoreski OV, Subramanian BR, et al. Human mammary epithelial cell transformation through the
33. Campbell PM, Groehler AL, Lee KM, et al. K-Ras promotes growth transformation and invasion of
immortalized human pancreatic cells by Raf and phosphatidylinositol 3-kinase signaling. Cancer Res
34. Levine BL, Cargile CB, Blazes MS, et al. PTEN mutations and microsatellite instability in complex
35. Sato M, Vaughan MB, Giraud L, et al. Multiple oncogenic changes (K-RAS(V12), p53 knockdown, mu-
tant EGFRs, p16 bypass, telomerase) are not sufficient to confer a full malignant phenotype on human bronchial
36. Zhao JJ, Gjoreski OV, Subramanian BR, et al. Human mammary epithelial cell transformation through the
37. Scott GR, Goga A, Bhauanik D, et al. Coordination of KRAS2 and PIK3CA-driven oncogenesis: combinatory

8136 www.aacrjournals.org

Acknowledgments

Received 2/28/2008; revised 6/22/2008; accepted 7/6/2008.

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.

We thank Robert A. Weinberg for HMLE cells and its subsline, Masato Nishida for Ishikawa-3 19·12 cell line, and Victor E. Velculescu for useful information. We thank Mayumi Kitagawa, Sang-Hyun Lee, Masashi Aonomu, Osamu Tetsu, Aibigail Miller, Frank Bos, Jesse Lyons, Anthony Karnezis, and Vivianne Wei Ding for helpful
discussions. We also thank Tetsu Yano, Toshiharu Tasugi, Shunsuke Nakagawa and Tomoyuki Neo for support and assistance, especially for organizing clinical samples and
data.

K. Oda: commercial research grant, Daiichi-Sankyo Pharmaceutical Co. Ltd.; speaker honoraria, Novartis; ownership interest, Onyx, Exelixis, and Neagenix. The other authors disclosed no potential conflicts of interest.

Cancer Research