High Frequency of Coexistent Mutations of *PIK3CA* and *PTEN* Genes in Endometrial Carcinoma

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Abstract

The phosphatidylinositol 3′-kinase (PI3K) pathway is activated in many human cancers. In addition to inactivation of the *PTEN* tumor suppressor gene, mutations or amplifications of the catalytic subunit α of PI3K (*PIK3CA*) have been reported. However, the coexistence of mutations in these two genes seems exceedingly rare. As *PTEN* mutations occur at high frequency in endometrial carcinoma, we screened 66 primary endometrial carcinomas for mutations in the helical and catalytic domains of *PIK3CA*. We identified a total of 24 (36%) mutations in this gene and coexistence of *PIK3CA/PTEN* mutations at high frequency (26%). *PIK3CA* mutations were more common in tumors with *PTEN* mutations (17 of 37, 46%) compared with those without *PTEN* mutations (7 of 29, 24%). Array comparative genomic hybridization detected 3q24-qter amplification, which covers the *PIK3CA* gene (3q26.3), in one of nine tumors. Knocking down PTEN expression in the HEC-1B cell line, which possesses both *K-Ras* and *PIK3CA* mutations, further enhances phosphorylation of Akt (Ser^{473}), indicating that double mutation of *PIK3CA* and *PTEN* has an additive effect on PI3K activation. Our data suggest that the PI3K pathway is extensively activated in endometrial carcinomas, and that combination of *PIK3CA/PTEN* alterations might play an important role in development of these tumors. (Cancer Res 2005; 65(23): 10669-73)

Introduction

The phosphatidylinositol 3′ -kinases (PI3K) are widely expressed lipid kinases that catalyze the production of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn contributes to the recruitment and activation of a wide range of downstream targets, including Akt (1). PTEN is a lipid phosphatase that counteracts the activity of PI3K (2). *PTEN* is frequently mutated in various tumors, including endometrial carcinoma (34-55%; refs. 3, 4). Recent studies showed an inverse correlation between loss of PTEN expression and Akt activation (5). Increased PI3K activity via gain of function has also been shown in a number of human cancers: *PIK3CA*, which encodes the catalytic subunit p110α of PI3K, is located on chromosome 3q26.3, and amplification of this locus was suggested to increase PI3K activity (6). Moreover, Samuels et al. identified somatic mutations of *PIK3CA* in several types of human tumors (7). Functional analysis revealed that several "hotspot" mutants of p110α (E542K, E545K, and H1047R) increase lipid kinase activity and induce oncogenic transformation (8). Simultaneous mutation in *PIK3CA* and *PTEN* has been thought to be mutually exclusive, as reported in breast carcinoma and glioblastoma (9, 10). However, in this study, we show the frequent coexistence of *PIK3CA* and *PTEN* mutations in endometrial carcinomas. We also show that *PIK3CA* copy number may change in endometrial carcinomas.

Materials and Methods

Tumor samples and genomic DNA. Surgical samples were obtained from 66 patients with primary endometrial carcinomas who underwent resection of their tumors at the University of Tokyo Hospital. All of the patients provided informed consent for the research use of their samples and the collection, and use of tissues for this study was approved by the appropriate institutional ethics committees. Genomic DNA was extracted by a standard SDS-proteinase K procedure. The clinical status was described previously (4). Detailed distribution of the histologic subtypes was as follows: 58 (88%) endometrioid adenocarcinomas, three adenosquamous carcinomas, one clear cell carcinoma, one squamous cell carcinoma, and three mixed carcinomas.

PCR and sequencing. Primer sequences and PCR conditions of exons 9 and 20 of *PIK3CA* have been described previously (7). PCR products were sequenced with the BigDye terminator method (Applied Biosystems, Foster City, CA) on an autosequencer (ABI PRISM 3700).

Statistical analysis. Survival curves were constructed using the Kaplan-Meier method and compared with a log-rank test. The analyses were carried out using the JMP 5.11J statistics package (SAS Institute, Cary, NC). The association of variables was evaluated by the Fisher's exact test. *P*s obtained in all tests were considered significant at $P < 0.05$.

Cell lines. AN3CA, KLE, HEC-1B, and RL95-2 were obtained from the American Type Culture Collection (Manassas, VA). Ishikawa3-H-12 was a generous gift of Dr. Masato Nishida (Kasumigaura Medical Center, Ibaraki, Japan). Ishikawa3-H-12, AN3CA and HEC-1B cells were maintained in Eagle's MEM with 10% fetal bovine serum (FBS). KLE and RL95-2 cells were grown in 1:1 mixture of DMEM and Ham's F12 medium with 10% FBS. We confirmed *PTEN* and *K-Ras* mutations (entire coding lesion of *PTEN* and exons 1 and 2 of *K-Ras*) by PCR and sequencing, using genomic DNA or cDNA of each cell line. Only HEC-1B possesses *K-Ras* mutation (G12D). *PTEN* mutation was detected in AN3CA [codon 130 and 1-bp (G) del], Ishikawa3-H-12 [codon 289, 1-bp (A) del and codons 318-319, 4-bp (CTTA) del], and RL95-2 [codon 322, 1-bp (A) del and codon 322, 1-bp (A) ins].

Western blotting. Cells were lysed in 500 μL of buffer A [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L sodium chloride, 5 mmol/L EDTA, 2 mmol/L sodium orthovanadate, and 10 mmol/L sodium fluoride] containing 1% Triton X-100. Western blotting was done with 15 μg of each extract and antisera specific for PTEN (138G6; Cell Signaling, Beverly, MA, 1:1,000), phospho-Akt (Ser^{473}; Cell Signaling; 1:1,000), phospho-PDK1 (Ser^{241}; Cell Signaling; 1:1,000), phospho-GSK3β (Ser^{9}; Cell Signaling; 1:1,000), β-actin (Sigma, St. Louis, MO; 1:5,000), and K-Ras (Santa Cruz Biotechnology, Santa Cruz, CA; F234; 1:500) in TBST/0.5% milk followed by anti-rabbit-horseradish peroxidase (HRP; Amersham Biosciences, Piscataway, NJ; 1:5,000) or anti-mouse HRP (Amersham; 1:5,000). Immunoreactive proteins were detected by chemiluminescence (Enhanced Chemiluminescence Plus, Amersham) and autoradiography.

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

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Table 1. *PIK3CA* mutations in endometrial cancer

Exon	Nucleotide	Amino acid	Number
9	G1624A	E542K	1
9	G1624C	E542Q	1
9	G1633A	E545K	1
9	A1634G	E545G	1
20	G3019C	G1007R	1
20	T3061C	Y1021H	1
20	A3062C	Y1021C	1
20	C3104T	A1035V	1
20	G3129T	M1043I	1
20	C3139T	H1047Y	5
20	A3140G	H1047R	6
20	G3149A	G1050D	1
20	C3155A	T1052K	1
20	A3194T	H1065L	1
20	A3207G	Stop 1069 Ins 4aa (WKDN)	1

Small interfering RNA transfection. Small interfering RNA (siRNA) was used to inhibit the expression of the *K-Ras* or *PTEN* gene in HEC-1B cell line. The targeted sequence of K-Ras siRNA and PTEN siRNA is 5′-AACCTGTCTC-TTGGATATTCT-3′ and 5′-AACAGTAGAGGAGCCGTCAAA-3′, respectively. Cells were seeded at 2.0×10^5 per six-well plate 24 hours before transfection and transfected with 80 or 160 nmol/L siRNA duplexes, using Lipofect-AMINE 2000 (Invitrogen, Carlsbad, CA). After 6 hours of transfection, the growth media were replaced to Eagle's MEM with 10% FBS. Cells were collected 72 hours after transfection and analyzed by immunoblotting.

Array comparative genomic hybridization. Array comparative genomic hybridization (array CGH) was carried out using arrays of 2464 BAC clones each printed in triplicate (HumArray2.0) according to published protocols (11). The tumor genomic DNA and normal male reference DNA (300 ng each) were labeled by random priming in separate 50 μL reactions to incorporate Cy3 and Cy5, respectively. For each tumor, the data are plotted as the mean log 2 ratio of the triplicate spots for each clone normalized to the genome median log 2 ratio. The clones are ordered by position in the genome beginning at 1p and ending with Xq.

Results and Discussions

We have analyzed mutations in exons 9 and 20 of *PIK3CA* gene in 66 endometrial carcinoma patients. We specifically examined these two exons, because four fifths of the mutations of *PIK3CA* gene are clustered in these regions (7). Direct sequencing identified mutations in 24 of 66 (36%) patients, as summarized in Table 1. These data suggest that the incidence of *PIK3CA* mutation in endometrial carcinoma is one of the highest among all tumors examined. Most of the mutations were identified in exon 20 (20 of 24, 83%). All of these mutations except one were missense mutations, and the most common mutation detected in this study was H1047R (Table 1), in agreement with previous reports (10, 12, 13).

Coexistence of loss of PTEN expression and *PIK3CA* mutation has been rarely detected in other cancers (9, 10). However, we identified coexistence of *PTEN* and *PIK3CA* mutations in 26% (17 of 66) of patients, making use of the data of previously published *PTEN* mutations (4). Figure 1*A* shows the sequences of a tumor in which both genes are mutated. Tumors with *PTEN* mutation showed a tendency to carry *PIK3CA* mutation more frequently (17 of 37, 46%) than tumors without *PTEN* mutation (7 of 29, 24%), although statistical significance is not reached ($P = 0.078$ in Fisher's exact test). Subsequently, we evaluated the relationship between *PIK3CA* mutation and other clinicopathologic factors. There was no evidence of an association of *PIK3CA* mutations with histologic grade, International Federation of Gynecology and Obstetrics (FIGO) stage, lymph node metastasis, and estrogen/progesterone receptor status (Supplementary Data 1). These data are in striking contrast

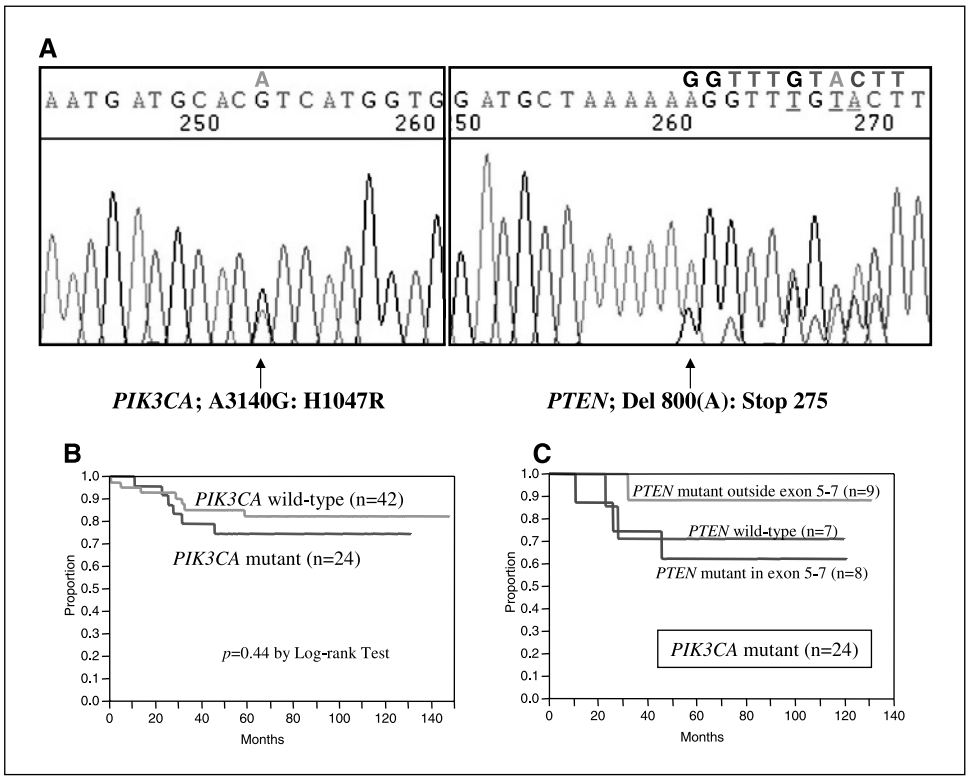

Figure 1. *A,* coexistence of *PIK3CA* and *PTEN* mutations. Sequence traces of one case for exon 20 of *PIK3CA* (*left*) and exon 7 of *PTEN* (*right*). *B, PIK3CA* mutational status and survival in endometrial carcinomas calculated according to Kaplan-Meier method. Survival of patients with *PIK3CA* mutations ($n = 24$) was compared with those with wild-type *PIK3CA* ($n = 42$). *C,* survival of *PIK3CA* mutant patients with *PTEN* mutations only outside exons 5 to 7 ($n = 9$) compared with those with *PTEN* mutations in exon 5, 6, or 7 ($n = 8$) or those with wild-type *PTEN* ($n = 7$).

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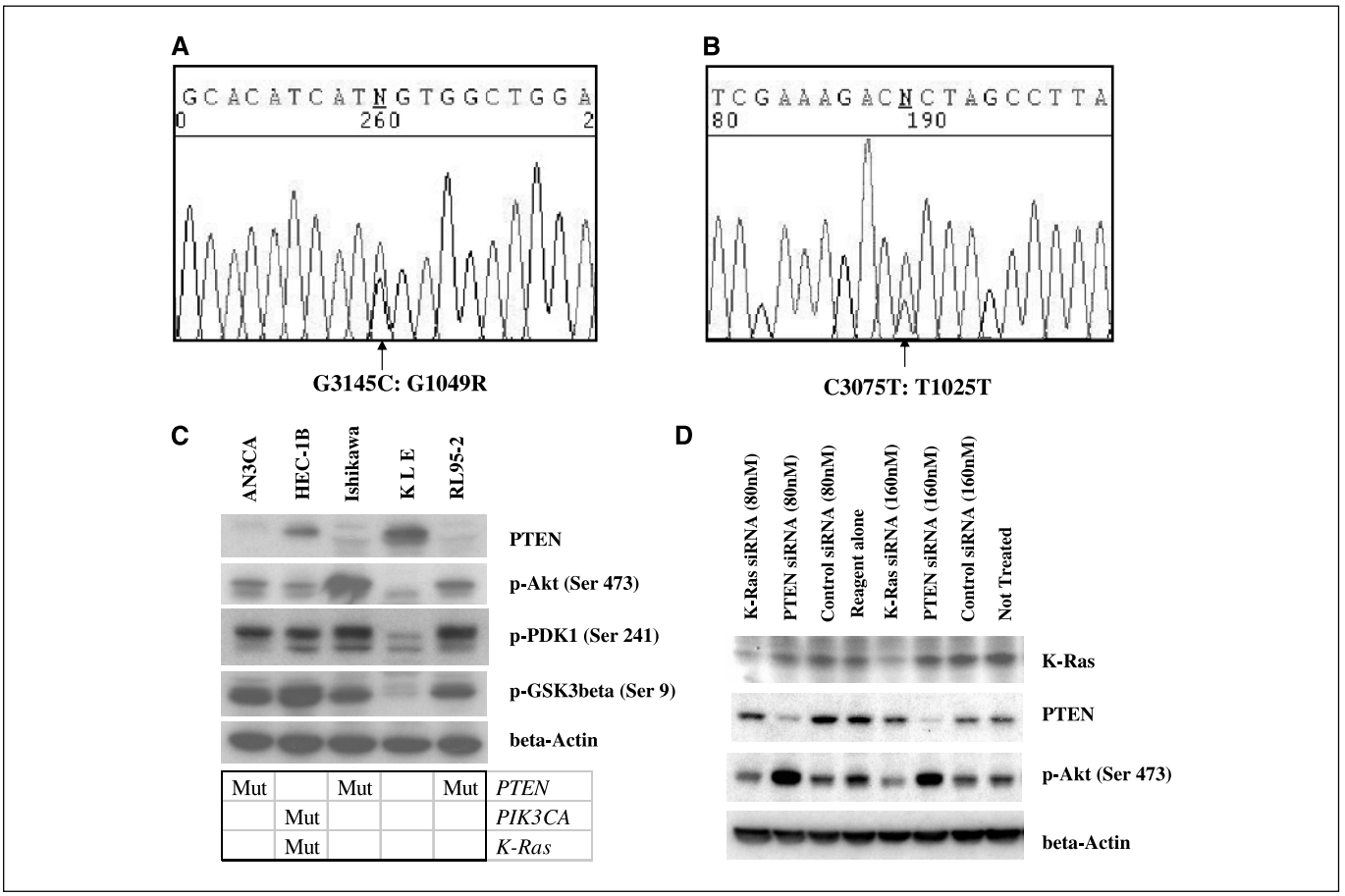

Figure 2. *A, PIK3CA* missense mutation in HEC-1B endometrial carcinoma cell line. *B, PIK3CA* silent mutation (no amino acid substitution) in Ishikawa 3-H-12 endometrial carcinoma cell line. *C,* phosphorylation of Akt (Ser^{473}), PDK1 (Ser^{241}), and GSK3β (Ser^{9}) is correlated with mutational status of *PTEN, PIK3CA*, and *K-Ras*. Cell lysates were analyzed by immunoblotting using specific antibodies. *Bottom,* status of *PTEN, PIK3CA*, and *K-Ras* in each cell line. *Mut,* mutant; *blank,* wild type. *D,* silencing K-Ras or PTEN by siRNA in HEC-1B cells. HEC-1B cells were transfected with 80 or 160 nmol/L siRNA duplexes targeted against K-Ras or PTEN. siRNA duplexes that do not match known genes were used as a control with 80 or 160 nmol/L [control (nonsilencing) siRNA; Qiagen, Inc., Valencia, CA]. Lysates of cells were immunoblotted with the indicated antibodies.

to those of breast carcinoma, showing that *PIK3CA* mutations correlate with expression of hormone receptors and node metastasis and are mutually exclusive with loss of PTEN expression (10). However, two of eight (25%) *PTEN* mutant breast carcinomas also possessed *PIK3CA* mutations, suggesting that coexistence of *PTEN/PIK3CA* mutations could occur in other tumors as well.

Next, we analyzed prognosis according to *PIK3CA* and *PTEN* status. Kaplan-Meier analysis suggested that *PIK3CA* mutation itself was not a marker of poor prognosis (Fig. 1*B*), although the sample size is small. Moreover, *PIK3CA* mutation did not affect the prognostic difference caused by *PTEN* mutational status (Fig. 1*C*). The group with *PTEN* mutation only outside exons 5 to 7 and *PIK3CA* mutation still showed better prognosis compared with the other groups with *PIK3CA* mutation. These results suggest that activated PI3K pathway alone is not a poor prognostic factor in endometrial carcinoma. The disparity of prognosis according to *PTEN* status, regardless of *PIK3CA* status, might indicate that *PTEN* mutation is not only associated with the PI3K pathway but also with other pathways.

Mutations in both *PTEN* and *PIK3CA* are most unexpected, because loss of PTEN and activation of PI3Ks are thought to have similar effects on the PIP3 pool. We could not find any correlations between types of *PIK3CA* mutations, such as H1047R or H1047Y, and *PTEN* mutations. Furthermore, previous clonal analyses showed that adenocarcinomas of uterine endometrium are monoclonal in composition (14). We propose three hypotheses to account for this controversial discovery. First, more than one input activating the PI3K/Akt pathway is required to completely activate this pathway. In breast carcinoma, *PIK3CA* mutations correlate with ErbB2 overexpression, suggesting that another activating event might be necessary to fully activate the PI3K pathway (10). The second hypothesis is that either PTEN or p110α possesses additional function(s) distinct from the PI3K pathway. In support of this, lipid phosphatase independent role for PTEN, including protein phosphatase activity, have been reported. Raftopoulou et al. showed that PTEN can inhibit cell migration through its C2 domain, depending on the protein phosphatase activity (15). Freeman showed that PTEN can interact with p53 and modulate p53 function independently of its phosphatase activity (16). Okumura et al. reported that the PTEN COOH-terminal domain physically interacts with the oncogenic MSP58 protein (17). These findings suggest that impairment of other PTEN function(s) might play a key role in endometrial carcinoma. Finally, other isoforms of p110 might have important roles in endometrial carcinogenesis. Mammals have three genes for the class IA p110 subunits encoding p110α, p110β, and p110δ and one gene for the class IB p110 subunit encoding

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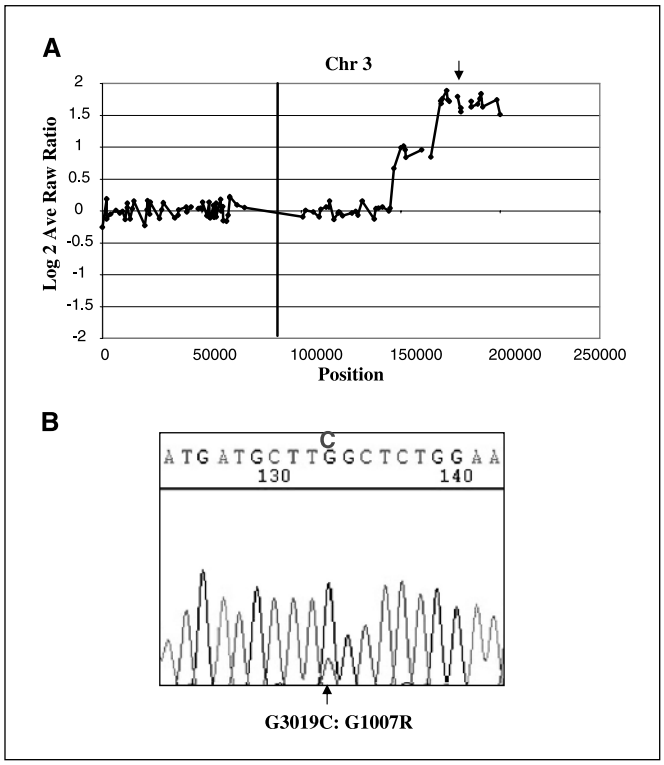

Figure 3. *A,* array CGH copy number profile of chromosome 3, showing amplification at 3q24-qter. *Arrow,* locus of *PIK3CA* (3q26.3). *B, PIK3CA* missense mutation in the case with *PIK3CA* amplification and *PTEN* mutations.

p110γ. Of them, α and β isoforms are widely expressed. Analysis of knockout mice or microinjection studies with neutralizing antibodies show different phenotypes among these isoforms (18), suggesting that they may have distinct cell type–specific functions.

To address the first hypothesis that more than one input is necessary to completely activate PI3K pathway, we analyzed five endometrial cancer cell lines. Among them, HEC-1B showed *PIK3CA* missense mutation in exon 20 (G3145C:G1049R) and Ishikawa3-H-12 showed a silent mutation in exon 20 (Fig. 2*A* and *B*). The status of *PTEN, PIK3CA*, and *K-Ras* is summarized in Fig. 2*C*. *K-Ras* mutation is also known to activate PI3K, and Akt, PDK1, and GSK3β are phosphorylated in response to activation of PI3K. As expected, PTEN expression was only detected in PTEN wild-type cells (HEC-1B and KLE), and Akt, PDK1, and GSK3β were clearly phosphorylated in four cell lines except for KLE cells, which did not show any mutations in *K-Ras, PIK3CA*, and *PTEN* (Fig. 2*C*). To investigate whether *PIK3CA* mutation alone could activate PI3K pathway, we knocked down K-Ras by transfecting siRNA in HEC-1B cells. Figure 2*D* shows that Akt was still phosphorylated in these cells in spite of the decreased K-Ras expression. Samuels et al. also reported that endogenous mutant p110α could increase the level of phospho-Akt in colon cancer cells (19). Next, we addressed the effect of having both *PTEN* and *PIK3CA* alterations by knocking down PTEN in HEC-1B cells. Figure 2*D* shows that knock down of PTEN protein further increased the level of phospho-Akt. These data suggest that either loss of PTEN function or *PIK3CA* mutation can independently activate PI3K pathway, and that double mutation of *PTEN* and *PIK3CA* could enhance the activity more efficiently.

Amplification of *PIK3CA* (3q26.3) has been shown to increase PI3K activity in ovarian cancer (6). Recently, 3q26 was also shown to be frequently amplified in endometrial carcinomas, especially in poorly differentiated or serous adenocarcinomas (20). We therefore did array CGH in 9 of 66 patients. Three cases showed significant chromosome changes (data not shown). Of them, one sample showed significant amplification at 3q24-qter (Fig. 3*A*). The histology of this tumor was mixed (endometrioid, serous, and clear) adenocarcinoma, compatible with the previous report (20). Interestingly, we found both *PIK3CA* (G3019C;G1007R) and *PTEN* [C388G;R130G and codon 267; 1-bp (A) del] mutations in this sample. Figure 3*B* showed that the level of mutant band (C) of *PIK3CA* is much lower than that of the normal band (G), indicating that *PIK3CA* mutation occurred in nonamplified allele. Campbell et al. previously described inverse association of the presence of *PIK3CA* mutation and gene amplification in ovarian cancers (12). The lower frequency of *PIK3CA* amplification (one of nine samples) and the occurrence of *PIK3CA* mutation in nonamplified allele might support their findings about the mutual exclusion. Our data showed that amplification of *PIK3CA* could also coexist with *PTEN* mutation in endometrial carcinomas.

In conclusion, we identified *PIK3CA* mutation in 36% of endometrial carcinomas. In particular, *PIK3CA, PTEN* double mutations occur at high frequency (26%) in endometrial carcinomas. Suppressing PTEN expression could further enhance the phosphorylation of Akt in HEC-1B cells with *K-Ras* and *PIK3CA* mutations. Moreover, we showed that amplification of *PIK3CA* locus could be also detected in endometrial carcinomas. We believe that additional extensive studies on *PTEN* and *PIK3CA* in endometrial carcinoma will help clarify more function of these two genes, as well as the function in PI3K pathway.

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