Short Communication

Frequent Activating Mutations of *PIK3CA* in Ovarian Clear Cell Carcinoma

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Ovarian clear cell carcinoma (CCC) is one of the most malignant types of ovarian carcinomas, particularly at advanced stages. Unlike the more common type of ovarian cancer, high-grade serous carcinoma, ovarian CCC is often resistant to platinum-based chemotherapy, and therefore an effective treatment for this tumor type at advanced stages is urgently needed. In this study, we analyzed 97 ovarian CCCs for sequence mutations in *KRAS*, *BRAF*, *PIK3CA*, *TP53*, *PTEN*, and *CTNNB1* as these mutations frequently occur in other major types of ovarian carcinomas. The samples included 18 CCCs for which affinity-purified tumor cells from fresh specimens were available, 69 microdissected tumors from paraffin tissues, and 10 tumor cell lines. Sequence mutations of *PIK3CA*, *TP53*, *KRAS*, *PTEN*, *CTNNB1*, and *BRAF* occurred in 33%, 15%, 7%, 5%, 3%, and 1% of CCC cases, respectively. Sequence analysis of *PIK3CA* in 28 affinity-purified CCCs and CCC cell lines showed a mutation frequency of 46%. Samples with *PIK3CA* mutations showed intense phosphorylated AKT immunoreactivity. These findings demonstrate that ovarian CCCs have a high frequency of activating *PIK3CA* mutations. We therefore suggest that the use of *PIK3CA*-targeting drugs may offer a more effective therapeutic approach compared with current chemotherapeutic agents for pa-

tients with advanced-stage and recurrent CCC. (Am J Pathol 2009, 174:1597–1601; DOI: 10.2353/ajpath.2009.081000)

Ovarian carcinomas are a heterogeneous group of neoplasms that can be classified according to the type and degree of differentiation. Despite the fact that clinical management of ovarian cancer at the present time largely fails to take this heterogeneity into account, it has become clear that each major histological type has unique molecular genetic defects that deregulate specific signaling pathways in the cancer cells.¹ Ovarian clear cell carcinoma (CCC) is one of the most lethal types of ovarian cancer, with a 5-year survival rate (all stages) of less than 35%.² Unlike the more common type of ovarian carcinoma, high-grade serous carcinoma, CCC is often resistant to platinum-based chemotherapy.^{3–8} Several studies have attempted to elucidate the molecular pathogenesis of ovarian CCC with the goal of identifying molecular targets that are altered in this tumor type,¹ but these reports have been of limited value because of insufficient sample size.

In this study, we analyzed 97 ovarian CCCs for sequence alterations in genes that participate in several major cancer-associated pathways including *TP53*, *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, and *CTNNB1*. We demonstrated frequent *PIK3CA* mutations in CCCs, especially those from purified tumors and cell lines. Our findings suggest that *PIK3CA*-targeting drugs may be a more effective therapy than current chemotherapeu-

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Table 1. PCR and Sequencing Primers

Gene	Exon	Primer 1 sequence	Primer 2 sequence
<i>KRAS</i>	1	5'-GTAAACGACGGCCAGTTTGAACCCAAAGGTACATTTCAG-3'	5'-TCTTAAGCGTCGATGGAGGAG-3'
<i>KRAS</i>	2	5'-GTAAACGACGGCCAGTATGCATGGCATTAGCAAAGAC-3'	5'-CGTCATCTTTGGAGCAGGAAC-3'
<i>BRAF</i>	15	5'-GTAAACGACGGCCAGTTTGTGAATCTGGGAACATATGAAA-3'	5'-TCATCCTAACACATTTCAGCC-3'
<i>TP53</i>	3	5'-GTAAACGACGGCCAGTGGGAGATCCCAAGTTCCAAAC-3'	5'-ACGTTCTGGTAAGGACAAGGG-3'
<i>TP53</i>	4	5'-GTAAACGACGGCCAGTGGGCCAGACCTAAGAGCAATC-3'	5'-AAGCTCCTGAGGTGTAGACGC-3'
<i>TP53</i>	5	5'-AGGCCCTTAGCCTCTGTAAGC-3'	5'-GTAAACGACGGCCAGTCTGCTCAGATAGCGATGGTG-3'
<i>TP53</i>	6	5'-GTAAACGACGGCCAGTAGAAATCGGTAAGAGGTGGGC-3'	5'-CATCCTGGCTAACGGTGAAAC-3'
<i>TP53</i>	7	5'-GTAAACGACGGCCAGTTTGGGCAGTGTAGGAAAGAG-3'	5'-GTTGGAGTAGATGGAGCCTG-3'
<i>PIK3CA</i>	1	5'-TGCTTTTGGGACAACCATACATC-3'	5'-CTTGCTTCTTAAATAGTTCATGCTTT-3'
<i>PIK3CA</i>	1	5'-CCCCTCCATCAACTTCTTCAA-3'	5'-ATTGTATCATACCAATTTCTCGATTG-3'
<i>PIK3CA</i>	9	5'-TCAGCAGTTACTATTCTGTGACTGG-3'	5'-GTAAACGACGGCCAGTTGCTGAGATCAGCCAAATTC-3'
<i>PIK3CA</i>	20	5'-GTAAACGACGGCCAGTGACATTGAGCAAGACCTGAAG-3'	5'-TGGATTGTGCAATTCCTATGC-3'
<i>PTEN</i>	1	5'-TTTCCATCCTCGAAGAGAC-3'	5'-GTAAACGACGGCCAGTTCCGTCTAGCCAAACACACC-3'
<i>PTEN</i>	2	5'-GTAAACGACGGCCAGTTCTGTGATGTATAAACCGTGAGTTTC-3'	5'-CCCTGAAGTCCATTAGGTACGG-3'
<i>PTEN</i>	3	5'-GTAAACGACGGCCAGTCATGATTACTACTCTAAACCCATAGAAG-3'	5'-TCAAATATGGGCTAGATGCCA-3'
<i>PTEN</i>	4	5'-ATAAGATTTCAGCAATGTTTGTAG-3'	5'-GTAAACGACGGCCAGTGACCACTGCCTCAAATAGTAGG-3'
<i>PTEN</i>	5	5'-TGCAACATTTCTAAAGTTACCTACTTG-3'	5'-GTAAACGACGGCCAGTTTCTACTTGTCATTACACCTCAATAA-3'
<i>PTEN</i>	6	5'-AATGGCTACGACCCAGTTACC-3'	5'-GTAAACGACGGCCAGTTTGGCTTCTTTAGCCCAATG-3'
<i>PTEN</i>	7	5'-GTAAACGACGGCCAGTTGCAGATACAGAAATCCATATTTCG-3'	5'-AATGTCTCACCATTGCCAGAG-3'
<i>PTEN</i>	8	5'-TGCAACAGATAACTCAGATTGCC-3'	5'-GTAAACGACGGCCAGTTGTCAAGCAAGTTCTTCATCAGC-3'
<i>PTEN</i>	9	5'-GTAAACGACGGCCAGTAAAGATCATGTTTGTACAGTGCTTAAA-3'	5'-TGACACAATGTCTATTGCCA-3'
<i>CTNNB1</i>	2	5'-AAATATTTCAATGGGTTCATATACAG-3'	5'-GTAAACGACGGCCAGTTCCACAGTTCAGATTACCTAAG-3'

tic agents for patients with advanced stage and recurrent disease.

Materials and Methods

Tissue Specimens

The tumors included 10 cases from the Johns Hopkins Hospital, 52 cases from National Taiwan University Hospital, and 25 cases from the Seirei Mikatahara Hospital, Japan. H&E-stained sections from tissue specimens were reviewed and the diagnosis of ovarian CCCs confirmed by an expert gynecologic pathologist (RJK). All of the specimens were anonymous and tissues collected in compliance with institutional review board regulations. In addition, we also analyzed 10 established ovarian CCC cell lines. As the sensitivity of mutation detection in primary tumors is dramatically affected by the purity of the tumor DNA samples analyzed, we affinity purified tumor cells using Epi-CAM antibody magnetic beads from all 18 freshly collected samples.⁹ We also microdissected tumor cells from 69 paraffin-embedded tumors.

Mutational Analysis

The relevant exons of the genes indicated in Table 1 in each tumor sample were PCR-amplified, sequenced, and assessed for potential sequence alterations using approaches previously described.^{9,10} The nucleotide sequences were then analyzed using the Mutation Surveyor program (Soft Genetics LLC, State College, PA) and the sequencing data were analyzed by two investigators independently.

Immunohistochemistry

We assessed AKT phosphorylation in 58 CCCs including 18 cases with *PIK3CA* mutations and 40 cases without the mutations using an anti-pAkt (Ser473) monoclonal antibody (Cell Signaling). Immunohistochemistry was performed on deparaffinized sections using the antibody at a

dilution of 1:50 and an EnVision+System peroxidase kit (DAKO, Carpinteria, CA). Immunoreactivity was scored by two investigators as follows: 0: undetectable, 1+: weakly positive, 2+: moderately positive and 3+: intensely positive.

Single Nucleotide Polymorphism Array Analysis

Single nucleotide polymorphisms (SNPs) were genotyped using the 250K Styl arrays (Affymetrix, Santa Clara, CA) in the Microarray Core Facility at the Dana-Farber Cancer Institute, Boston, MA. The dChip 2006 program was used to analyze SNP array data.^{11,12} Data were normalized to a baseline array with median signal intensity at the probe intensity level using the invariant set normalization method. Signal values for each SNP were compared with the average intensities from 15 normal samples. To infer the DNA copy number from the raw signal data, we used the Hidden Markov Model,¹¹ based on the assumption of diploid for normal samples. Mapping information of SNP locations and cytogenetic bands were based on curation of Affymetrix and University of California Santa Cruz hg17. In this study, we used an arbitrary cutoff of more than three copies in more than six consecutive SNPs, to define an amplification.

Quantitative Real-Time PCR

DNA copy number of the *PIK3CA* locus in 10 CCC cell lines was assessed by quantitative real-time PCR using an iCycler (Bio-Rad, Hercules, CA) with SYBR green dye (Molecular Probes, Eugene, OR). The primer sequences that amplified the first exon are: 5'-CCCCTCCATCAACTTCTTCAA-3' (forward) and 5'-ATTGTATCATACCAATTCTCGATTG-3' (reverse).

Averages in the threshold cycle number of triplicate measurements were obtained. The results were expressed as the difference between the threshold cycle of the gene of interest and the threshold cycle of a *Line-1* gene for which expression is relatively constant among tumor tissues.

Table 2. Sequence Mutation Rates in Ovarian Clear Cell Carcinomas

Sample (n)	KRAS	BRAF	TP53	PIK3CA	CTNNB1	PTEN
JH (10)	20%	0%	10%	50%	0%	0%
TW (8)	0%	0%	ND	50%	0%	0%
Cell line (10)	10%	10%	20%	40%	0%	10%
TW-paraffin (44)	9%	0%	ND	25%	2%	ND
JP-paraffin (25)	0%	0%	ND	32%	8%	ND
Overall: non-paraffin (JH + TW + Cell line)	11%	3%	15%	46%	0%	5%
Overall (97)	7%	1%	15%	33%	1%	5%

JH: freshly purified tumor cells from tumor tissues at the Johns Hopkins Hospital.
 TW: freshly purified tumor cells from tumor tissues at the National Taiwan University Hospital.
 TW-paraffin: paraffin-embedded tumors from National Taiwan University Hospital.
 JP-paraffin: paraffin-embedded tumors from the Seirei Mikatahara Hospital, Japan.
 ND: not determined.

Results and Discussion

The mutation profile of all of the specimens is summarized in Table 2 and the detailed mutations in each sample were listed in supplementary Table 1 (at <http://ajp.amjpathol.org>). Sequence mutations (at the mutation hot spots) of *PIK3CA*, *TP53*, *KRAS*, *PTEN*, *CTNNB1*, and *BRAF*, were detected in 33%, 15%, 7%, 5%, 3%, and 1%, of informative CCC cases, respectively. Among the genes analyzed, *PIK3CA* was the most frequently mutated and was therefore selected for further characterization. We found that the percentage of *PIK3CA* mutations was even higher (46%) in the 28 cases of affinity purified CCCs and CCC cell lines. Most of the *PIK3CA* mutations were mapped to exon 9 and exon 20 resulting in kinase activation of p110 α which has been shown to result in increased cellular survival and invasion. Because AKT activation by phosphorylation can occur as a result of constitutive activating mutations in *PIK3CA*, we assessed AKT phosphorylation in 58 tumors, including 18 cases with *PIK3CA* mutations and 40 cases without mutations. We found that all 18 specimens with *PIK3CA* mutations and 34 (85%) of 40 cases with wild-type *PIK3CA* showed intense and diffuse phosphorylated AKT immunoreactivity. There was no statistical difference of phosphorylated AKT immunoreactivity (extent and intensity) between these two groups ($P = 0.16$). These results indicate that *PIK3CA* mutations directly activate AKT in CCCs, and other mechanisms rather than sequence mutations are involved in activating the pathway for those CCCs with wild-type *PIK3CA*.

In an effort to determine whether the *PIK3CA* locus was genomically amplified, we applied a genome-wide analysis for DNA copy number alterations in the 12 affinity-purified ovarian CCCs using 250K SNP arrays and performed quantitative real-time PCR to determine the DNA copy number in the *PIK3CA* locus in 10 cell lines. We did not observe any DNA copy number change in any of the tumors and cell lines (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Thus, it is likely that *PIK3CA* mutation is the main molecular genetic change that constitutively activates the PI3K/AKT pathway in ovarian CCC. Finally, to determine the clinical significance of *PIK3CA* mutation in ovarian CCCs, we correlated its mutation status with various clinicopathologic parameters. There was no significant correlation between the *PIK3CA* muta-

tion status and histological features, clinical stage, patient age, disease-free interval, and overall survival ($P > 0.05$ for all clinical parameters examined).

The findings from this study have important biological and clinical ramifications for ovarian cancer. First, although previous reports have detected *PIK3CA* mutations in ovarian CCC, those studies analyzed a small number of cases and therefore the investigators could not draw firm conclusions about the frequency of *PIK3CA* mutations and the generalizability of their findings.^{13–15} In the present study, we analyzed a total of 97 ovarian CCCs and demonstrated that more than a third had *PIK3CA* mutations. This observation is important because of the well-established role of PI3K in oncogenesis in several types of human cancer.^{16–19} It is particularly interesting that among all of the cancer types reported so far, ovarian CCC has the highest frequency of *PIK3CA* mutations. Of note, in the current study we only sequenced those exons with the most common mutations in the selected genes and therefore the actual mutation frequency could be higher than the one reported here.

Our results also provide additional support to the view that ovarian cancer is a heterogeneous group of neoplastic diseases, which are characterized by their signature molecular genetic aberrations.²⁰ Specifically, high-grade serous carcinoma is characterized by frequent mutations of *TP53* and high levels of chromosomal instability.^{20,21} In contrast, low-grade serous carcinoma frequently harbors

Figure 1. The mutation profile of *TP53*, *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, and *CTNNB1* in different histological types of ovarian epithelial neoplasms. The frequency of individual mutations is shown in the bar chart in various types of ovarian carcinoma including high-grade (HG) serous carcinoma, low-grade (LG) serous carcinoma, clear cell carcinoma, endometrioid carcinoma (EMCA), and mucinous carcinoma. The mutation frequency is estimated from several studies based on a sizable sample size.^{1,9,10,14,15,29–37} The frequency of *PIK3CA* mutation in clear cell carcinoma is based on the current study showing 46% in purified tumors and cell lines. The mutation frequency of *PTEN* and *CTNNB1* has not been determined (ND) in low-grade serous carcinomas.

mutations in *KRAS/BRAF/ERBB2*, endometrioid carcinoma has *PTEN* and *CTNNB1* mutations in most of cases, and mucinous carcinoma has *KRAS* mutations in more than half of the cases^{1,20} (Figure 1). The high frequency of *PIK3CA* mutation and the low rate of mutations in *KRAS*, *BRAF*, *TP53*, *PTEN*, and *CTNNB1* indicate that CCCs have a different molecular signature from other surface epithelial tumors. Both ovarian endometrioid carcinoma and CCC are thought to develop from endometriosis,²² but endometrioid carcinoma has a high frequency of *PTEN* and *CTNNB1* mutations while CCC has a low frequency of mutations in these genes. Thus, our data provide evidence that the underlying genetic events in CCC and endometrioid carcinoma are inherently different despite the fact that the precursor lesion, endometriosis, is the same for both tumors.

From a therapeutic perspective, our results underscore the importance of carefully separating these different types of ovarian tumors in clinical trials evaluating different types of treatments. The relatively high frequency of *PIK3CA* mutations holds promise for new therapeutic approaches using small molecule inhibitors targeting PI3K. New PI3K targeting drugs, including GDC-0941,²³ NVP-BEZ235,^{24,25} PI-103,²⁶ and SF1126, a LY294002 prodrug,²⁷ have recently been developed and are being evaluated in clinical trials.^{23,25,28} In the future, it will be important to design clinical trials to evaluate the efficacy of such inhibitors by correlating clinical response with *PIK3CA* mutational status or pathway activation. If such studies show promising results, this would be an important step in the development of customized treatment for ovarian CCC at advanced stages.

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