Original contribution

Clinicopathologic and biological analysis of PIK3CA mutation in ovarian clear cell carcinoma☆☆☆

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Summary Somatic mutations of PIK3CA (phosphoinositide-3-kinase) have recently been shown playing an important role in the pathogenesis of ovarian clear cell carcinoma. In this study, the frequency of PIK3CA mutations and the relationship of PIK3CA mutations with clinicopathologic and biological variables were investigated in ovarian clear cell carcinomas from Japanese patients. Mutational analysis of PIK3CA was performed in 56 primary ovarian clear cell carcinomas from Japanese women. The relationship of these mutations with various clinicopathologic and biological variables (phosphorylated AKT and phosphorylated mTOR (mammalian target of rapamycin) expression by immunohistochemistry) was determined. To clarify the roles of PI3K/AKT activation in ovarian clear cell carcinomas harboring PIK3CA mutations, we inactivated the PI3K/AKT/mTOR pathway in ovarian carcinoma cells with LY294002, temsirolimus and NVP-BEZ235. Missense mutations of PIK3CA were found in 16 (28.6%) of 56 ovarian clear cell carcinomas, but no mutation was found in 15 ovarian high-grade serous carcinomas. PIK3CA mutations were significantly associated with a favorable overall survival of patients with ovarian clear cell carcinoma (P < .05). There was no significant association between PIK3CA mutations and phosphorylated AKT or phosphorylated mTOR immunointensity status. No relationship was found between PIK3CA mutation status and sensitivity to PI3K/AKT/mTOR inhibitors in ovarian clear cell carcinoma cells. No association of PIK3CA mutations was found between positive phosphorylated AKT and positive

Keywords:
Ovarian clear cell carcinoma; PIK3CA; KRAS; BRAF; Mutation

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1. Introduction

Ovarian carcinoma is the most lethal gynecologic malignancy [1,2], and its incidence has increased in the last decade. Over the last 2 decades, aggressive cytoreducive surgery and platinum-taxane chemotherapy have dramatically improved the survival rate [3-5]; however, the 5-year overall survival remains below 50%. Despite aggressive treatment, most patients eventually recur with chemoresistant disease. Of the 4 subtypes (serous, endometrioid, clear cell, and mucinous), the clear cell histology accounts for 25% of cases in Japan and 8% to 10% of cases in the United States [6,7]. Ovarian clear cell carcinoma carries a poor prognosis even at an early stage because it is commonly resistant to platinum-based chemotherapy. Therefore, targeted biological agents may be more effective than conventional cytotoxic drugs. A complete understand of the pathways driving tumor growth in ovarian clear cell carcinoma is essential to design novel targeted therapeutics.

Ovarian clear cell carcinoma is distinct from its serous counterpart on a morphologic and molecular basis. Unlike serous carcinomas, for which a precursor lesion has not been identified, clear cell carcinoma develops in a stepwise fashion proceeding from atypical endometriosis to an invasive carcinoma [8-11]. Mutations in PIK3CA [12] and ARID1A (the AT-rich interactive domain 1A) [13,14] and genomic amplification of chr20q13.2 [15] are the most common molecular genetic alterations identified in ovarian clear cell carcinoma.

The PI3K/AKT oncogenic signaling pathway is activated in many human epithelial cancers [16-19]. Its activation directly counteracts the action of the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10), a negative regulator of PI3K/AKT. Activated PI3K/ AKT regulates the expression of several downstream target genes that inhibit apoptosis and promote cell proliferation. The p110α catalytic subunit of PI3K (phosphatidylinositol 3 kinase), is an oncogene located on chromosome 3q26.3, which is mutated in several types of cancer [19]. PIK3CA mutations increase PI3K activity, cell survival, motility, and cell cycle progression. Mutations are usually missense and cluster in exons 9 (helical domain) and 20 (kinase domain). We have recently shown that PIK3CA mutations are present in 40% of ovarian clear cell carcinoma [12,13]. However, the role of PIK3CA mutations in ovarian clear cell carcinoma behavior has not been fully elucidated.

The present study analyzed the relationship between somatic mutations of PIK3CA (phosphoinositide-3-kinase) phosphorylated mTOR, which suggests that the PI3K/AKT/mTOR pathway may be activated by other molecular mechanisms. Although PIK3CA mutations were associated with a more favorable prognosis, they did not predict the sensitivity of ovarian clear cell carcinoma cells to PI3K/AKT/mTOR inhibitors. © 2012 Elsevier Inc. All rights reserved.

2. Materials and methods

2.1. Tissue samples

Formalin-fixed, paraffin-embedded tissue samples of 71 ovarian cancers including 56 clear cell carcinomas and 15 high-grade serous carcinomas were used in this study. Samples were obtained from the Department of Obstetrics and Gynecology at the Shimane University Hospital and the Department of Obstetrics and Gynecology at Seirei Hamamatsu General Hospital. Diagnosis was based on the conventional morphologic examination of sections stained with hematoxylin and eosin (H&E), and tumors were classified according to the World Health Organization classification. Tumor staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification. All patients were primarily treated with cytoreductive surgery and adjuvant platinum and taxane or CPT-11 chemotherapy cis-(1,1-cyclobutanedicarboxyloxy) diammineplatinum(II) [CBDCA] AUC5 with paclitaxel 175 mg/m² or docetaxel 70 mg/m² or CDDP [cisplatin] 60 mg/m² with CPT-11 [camptothecin-11] 180 mg/m²). All patients received 6 to 12 courses of this combination regimen.
Acquisition of tissue specimens and clinical information was approved by an institutional review board (Shimane University and Seirei Hamamatsu General Hospital). The paraffin tissue blocks were organized into tissue microarrays, each made by removing 3-mm-diameter cores of tumor from the block. Selection of the area to core was made by a gynecologic oncologist (K.N.) and pathology technician (K.I.) and was based on a review of the H&E slides.

### 2.2. Cell culture and cell lines

The human ovarian cancer cell lines SKOV3, A2780, MDAH2774 (serous carcinoma), TOV-21G, and ES2 (clear cell carcinoma) were obtained from the American Tissue Culture Center (Rockville, MD). The OVK#18 (serous carcinoma) human ovarian cancer cell line was obtained from Tohoku University (Sendai, Japan). The JHOC9 and JHOC5 (clear cell carcinoma) were obtained from Riken Biobank (Osaka, Japan). The ovarian clear cell carcinoma cell lines OVISE, OVMANA, OVTOKO, and RMG1 were obtained from the Japanese Health Science Research Resources Bank (Osaka, Japan). The OV207 (clear cell carcinoma) was a kind gift from Dr Vijayalakshmi Shridhar (Mayo Clinic, Rochester, MN).

### 2.3. Mutational analysis of PIK3CA, KRAS, and BRAF

Genomic DNA was purified from all of the cell lines and formalin-fixed, paraffin-embedded tissues using a Qiaquick polymerase chain reaction (PCR) purification kit (Qiagen, Valencia, CA). As a healthy control, we used the DNA purified from uterus of the same patients. PCR was then performed, followed by nucleotide sequencing using the iCycler (Bio-Rad, Rockford, IL). Exons 1, 9, and 20 of PIK3CA; exon 1 of KRAS; and exon 15 of BRAF were sequenced because these mutational hot spots, together, harbor nearly all published mutations [13,21-23]. The primers for PCR and sequencing were described in a previous report [24]. The sequences were analyzed using the Lasergene program, DNASTAR (Madison, WI).

### 2.4. Immunohistochemistry

Expression levels of the phosphorylated form of AKT and mTOR were assessed by immunohistochemistry. The antibodies were rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA) that reacted with p-AKT and p-mTOR. Immunohistochemistry was performed with these antibodies on tissue microarrays at dilutions of 1:100, followed by detection with the EnVision+ System using the peroxidase method (Dako). The evaluation criteria for Ki-67 has been detailed in a previous report [25]. Patients were stratified into 2 groups depending on the median value (40) of Ki-67 labeling index.

### 2.5. Western blot analysis

Cell lysates were prepared by dissolving cell pellets in Laemmli sample buffer (Bio-Rad) supplemented with 5% β-mercaptoethanol (Sigma, St Louis, MO). Western blot analysis was performed on ovarian cancer cell lines SKOV3, A2780, MDAH2774, ES2, OVK#18, JHOC9, JHOC5, OVISE, OVMANA, OVTOKO, RMG1, TOV-21G, and OV207. Similar amounts of total protein from each lysate were loaded and separated on 10% Tris–glycine–sodium dodecyl sulfate polyacrylamide gels (Novex, San Diego, CA) and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes (Billericia, MA). The membranes were probed with p-AKT antibody (1:1000; Cell Signaling Technology) or p-mTOR antibody (1:100; Cell Signaling Technology), followed by a peroxidase-conjugated anti-rabbit immunoglobulin (1:20,000). The same membrane was probed with an antibody that reacted with glyceraldehyde 3-phosphate dehydrogenase (1:10,000) (Cell Signaling Technology) for loading control. Western blots were developed by chemiluminescence (Pierce, Rockford, IL).

### 2.6. Cell growth assays

For the cell growth assay, cells were plated at the same density (3 × 10^5 cells/well) in 96-well plates. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell growth assay was performed [26] 96 hours after treating the cells with LY294002 (Calbiochem, La Jolla, CA), temsirolimus (Selleck Chemicals, Houston, TX), NVP-BEZ235 (Selleck Chemicals) at 5 μM and at 500 and 250 nM, respectively, or with dimethylsulfoxide (DMSO) (control). The data were expressed as a percentage of the DMSO control. The mean and SD were obtained from 3 experiments.

### 2.7. Statistical methods for clinical correlation

Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. The
3. Results

3.1. Identification of PIK3CA, KRAS, and BRAF mutations

Somatic mutations of PIK3CA were identified in 16 (28.6%) of 56 ovarian clear cell carcinoma samples (Table 1; Fig. 1). All PIK3CA mutations were mapped to exons 9 and 20.
Of the 56 ovarian clear cell carcinomas analyzed, 37 and 44 cases were available for DNA sequencing for \textit{KRAS} and \textit{BRAF}, respectively. Somatic mutations of \textit{KRAS} were identified in 2 (5.4\%) of 37 cases. In contrast, somatic mutations of \textit{BRAF} were identified in 0 (0\%) of 44 cases. Simultaneous mutations of \textit{PIK3CA} and \textit{KRAS} did not occur in the tested ovarian clear cell carcinomas (Table 1). The frequency of \textit{PIK3CA} mutations in ovarian high-grade serous carcinomas (0.0\%; 0/15) was significantly lower than that in clear cell carcinomas (28.6\%; 16/56) ($P_b .05$).

### 3.2. Relationship between \textit{PIK3CA} mutations and p-AKT or p-mTOR expression and clinicopathologic factors

The immunoreactivity of p-AKT was detected in both the nucleus and the cytoplasm of the tumor cells (Fig. 2). This is consistent with a previous report [28]. Positive p-AKT was identified in 26 (46.4\%) of 56 ovarian clear cell carcinoma samples. The patients were stratified into 2 groups depending on the mutation status of \textit{PIK3CA}. The relationships between \textit{PIK3CA} mutations and clinicopathologic factors including p-AKT and p-mTOR expression are shown in Table 2. There was no significant correlation between \textit{PIK3CA} mutations and FIGO stage, cancer antigen 125 levels, Ki-67 labeling index, or the status of residual tumor. \textit{PIK3CA} mutation was significantly correlated with younger age ($P = .0420$; Table 2). \textit{PIK3CA} mutation tended to be more frequent in tumors associated with endometriosis. However, the difference was not statistically significant ($P = .1760$).

<table>
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<th>Factors</th>
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Abbreviation: CA125, cancer antigen 125.
addition, there were no significant relationship between PIK3CA mutations and p-AKT (\(P = .3968\)) and p-mTOR expression (\(P = .0703\)), except the relationship between p-AKT expression and p-mTOR expression (\(P = .0124\)).

### 3.3. Prognostic effect of PIK3CA mutations and p-AKT or p-mTOR expression

Next, we examined the prognostic effect of PIK3CA mutations and p-AKT and p-mTOR expression (Fig. 3).

Interestingly, an activating mutation in PIK3CA correlated with favorable overall survival in patients with ovarian clear cell carcinoma treated with platinum-based chemotherapy (\(P = .0342\)). Activating mutation in PIK3CA tended to correlate with longer progression-free survival in patients with ovarian clear cell carcinomas treated with platinum-based chemotherapy. However, the difference was not statistically significant (\(P = .1033\)). There was no significant relationship between p-AKT expression and overall/progression-free survival (\(P = .4391\) and \(P = .3155\), respectively). There was a significant relationship between p-
mTOR expression and favorable progression-free survival but not overall survival (P = .0423 and P = .1833, respectively). In the univariate analysis, FIGO stages III and IV (P < .01), status of residual tumor (P < .01), and wild-type PIK3CA (P < .05) significantly predicted shorter overall survival of patients with ovarian clear cell carcinoma.

### 3.4. Effects of PI3K/AKT/mTOR inactivation on ovarian carcinoma in vitro

A panel of ovarian clear cell carcinoma and serous carcinoma cell lines were first analyzed for the mutational statuses of the PIK3CA, KRAS, and BRAF genes. As shown in Fig. 4A, 6 ovarian cancer cell lines harbored PIK3CA, KRAS, or BRAF mutations. Interestingly, mutational status was not correlated with p-AKT or p-mTOR expression levels in clear cell carcinoma cell lines. Western blot analysis showed a dose-dependent effect of the PI3K/AKT/mTOR inhibitors on the expression of active AKT or mTOR in JHOC9 cells (Fig. 4B). Active AKT or mTOR was not detectable 1 hour after treating the cells with LY294002, temsirolimus, or NVP-BEZ235 at a concentration of 5 μM and of 500 and 250 nM, respectively, except the p-AKT expression with temsirolimus. Furthermore, mutational status was not correlated with growth inhibition by any of the 3 inhibitors (Fig. 5). Treatment with the PI3K/AKT/mTOR inhibitors failed to inhibit proliferation (<50% of DMSO control) in 4 of the cell lines harboring PIK3CA mutations. In contrast, proliferation was inhibited in some cell lines with wild-type PIK3CA (Fig. 5). Cell viability after treatment with the PI3K/AKT/mTOR inhibitors was not impacted in the 3 ovarian cancer cell lines harboring either KRAS or BRAF mutations.

### 4. Discussion

According to our result, the frequency and location of the PIK3CA mutations were similar to those reported for white and Asian (Mixed Japanese and Taiwanese) cases of ovarian clear cell carcinoma [12,13]. These results indicate that the contribution of PIK3CA mutations to the pathogenesis and progression of ovarian clear cell carcinoma is probably similar between these ethnicities.

Our study found no significant association between PIK3CA mutation and p-AKT or p-mTOR expression. This is consistent with the study by Mori et al [27] that failed to find an association between PIK3CA mutation and p-AKT expression in endometrial cancer. These findings suggest that additional factors may be required for activating the PI3K/AKT pathway in ovarian clear cell carcinoma or that p110α has functions distinct from PI3K/AKT regulation.
In the present study, a positive correlation was observed between p-AKT and the expression of the downstream target p-mTOR. It may be the downstream targets such as mTOR that are involved in the carcinogenesis of ovarian clear cell carcinoma.

The prognostic significance of PIK3CA mutation was also addressed in the present study. The effect of PIK3CA mutation on tumor behavior may be mediated through the phosphorylation of AKT, which is a poor prognostic factor [28,29]. This association has been attributed to platinum resistance of p-AKT-positive tumors. Thus, we assumed that tumors with PIK3CA mutations would be associated with poor prognosis in the present study in which patients had been treated with platinum-based chemotherapy. Few studies have addressed the relationship between PIK3CA mutations and prognosis. Li et al [30] reported a significant association of PIK3CA mutations with poor prognosis. In contrast, our study as well as the prior study by Maruyama et al [31] reported a significant association of PIK3CA mutations with a favorable prognosis. This finding is
unexpected in that p-AKT positive tumors are associated with poor prognosis [28,29]. In PIK3CA-mutated tumors, the PI3K/AKT pathway is probably the principal pathway for carcinogenesis and progression; however, AKT is activated by several factors in addition to PIK3CA mutation (eg, EGFR/HER-2). Therefore, it is plausible that PIK3CA mutation status and AKT activation may impact tumor behavior differently.

PIK3CA mutation may be associated with a less aggressive phenotype. In the current study, PIK3CA mutation tended to be more frequent in tumors associated with endometriosis. Previously, we reported that ovarian clear cell carcinomas associated with endometriosis had a more favorable outcome in comparison with ovarian clear cell carcinomas without endometriosis [11]. Taken together, previous reports and the current results suggest that tumors with PIK3CA mutations may represent a more indolent subset of ovarian clear cell carcinoma.

Although the biological roles of the PI3K/AKT/mTOR pathways in human cancer have been thoroughly investigated, there have been no recent studies in ovarian clear cell carcinoma. It is not known whether these pathways mediate the effect of activating PIK3CA mutations on tumor progression of ovarian clear cell carcinoma. In this study, we analyzed the genotype-phenotype correlation of ovarian clear cell carcinoma cells using 3 different PI3K/AKT/mTOR inhibitors. Unexpectedly, PI3K catalytic α-subunit (PIK3CA) mutations did not sensitize cancer cells to PI3K/AKT/mTOR inhibitors. In addition, they showed that the concurrent presence of PIK3CA and KRAS mutations did not predict drug resistance. This is in contrast to a recent report demonstrating that PIK3CA and KRAS mutations predict the response to the mTOR inhibitor everolimus in colorectal and breast carcinomas [32]. This discrepancy may be due to differences in organ-specific oncogenic pathways. Recently, it has been reported that ARID1A inactivating mutations play an important role in ovarian clear cell carcinoma [33]. Furthermore, recent study showed that ARID1A cooperates with p53 to inhibit tumor growth, and mutations in the ARID1A and TP53 genes are mutually exclusive in ovarian clear cell carcinoma [34]. More recently, we reported that loss of ARID1A expression may affect chemosensitivity in ovarian clear cell carcinomas [35]. Thus, ARID1A mutation, which has been identified in approximately half of clear cell carcinoma cases [13,14], could be another possible reason for the lack of relationship between PIK3CA mutation status and sensitivity to PI3K/AKT/mTOR inhibitors.

In conclusion, we have identified somatic missense mutations of PIK3CA in 16 (28.6%) of 56 ovarian clear cell carcinomas from Japanese women. The mutations were not significantly associated with p-AKT– or p-mTOR–positive tumors. PIK3CA mutation status did not predict sensitivity to PI3K/AKT/mTOR inhibitors in ovarian clear cell carcinoma cells in vitro. Patients with PIK3CA-mutated tumors, however, showed a significantly more favorable overall prognosis than those with PIK3CA wild-type tumors. Because our findings were based on a retrospective analysis of a relatively small number of patients, a prospective study is required to confirm the role of PIK3CA mutation on the prognosis of ovarian clear cell carcinoma.

References


