Whole-genome sequencing revealed novel prognostic biomarkers and promising targets for therapy of ovarian clear cell carcinoma

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**Background:** Ovarian clear cell carcinoma (OCCC) is mostly resistant to standard chemotherapy that results in poor patient survival. To understand the genetic background of these tumours, we performed whole-genome sequencing of OCCC tumours.

**Methods:** Tumour tissue samples and matched blood samples were obtained from 55 Japanese women diagnosed with OCCC. Whole-genome sequencing was performed using the Illumina HiSeq platform according to standard protocols.

**Results:** Alterations to the switch/sucrose non-fermentable (SWI/SNF) subunit, the phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathway, and the receptor tyrosine kinase (RTK)/Ras signalling pathway were found in 51%, 42%, and 29% of OCCC tumours, respectively. The 3-year overall survival (OS) rate for patients with an activated PI3K/Akt signalling pathway was significantly higher than that for those with inactive pathway (91 vs 40%, hazard ratio 0.24 (95% confidence interval (CI) 0.10–0.56), \( P = 0.0010 \)). Similarly, the OS was significantly higher in patients with the activated RTK/Ras signalling pathway than in those with the inactive pathway (91 vs 53%, hazard ratio 0.35 (95% CI 0.13–0.94), \( P = 0.0373 \)). Multivariable analysis revealed that activation of the PI3K/Akt and RTK/Ras signalling pathways was an independent prognostic factor for patients with OCCC.

**Conclusions:** The PI3K/Akt and RTK/Ras signalling pathways may be potential prognostic biomarkers for OCCC patients. Furthermore, our whole-genome sequencing data highlight important pathways for molecular and biological characterisations and potential therapeutic targeting in OCCC.
Ovarian clear cell carcinoma (OCCC) is recognised in the World Health Organisation classification of ovarian tumours as a distinct histological entity that demonstrates a markedly unique clinical behaviour from the other epithelial ovarian cancers (Scully, 1975; Itamochi et al., 2008). The OCCC constitutes ~4–12% of all epithelial ovarian cancers in western countries and >20% in Japan (Itamochi et al., 2008). The OCCC was diagnosed twice as frequently (11.1%) among Asian women living in the United States that that among Caucasians (4.8%) (Chan et al., 2008). However, the reason for the ethnic differences of OCCC prevalence remains unknown. The poor prognosis of patients with advanced disease (median survival time 12.7 months) may reflect the resistance of CCC to conventional platinum- and taxane-based chemotherapy (Sugiyama et al., 2000; Itamochi et al., 2008). Recently, randomised phase III clinical trial of irinotecan plus cisplatin (CPT-P) compared with paclitaxel plus carboplatin (TC) in treating patients with CCC was conducted by the Japanese Gynecologic Oncology Group (JGOG) in collaboration with the Gynecologic Cancer Intergroup (GGIC; JGOG3017/GCIG Trial) (Sugiyama et al., 2016). However, no significant survival benefit was observed for CPT-P. Therefore, effective and novel treatment strategies (e.g., incorporating molecular targeted agents) are required to improve outcomes for women with advanced OCCC.

Previous molecular studies have shown that CCC had a variety of genetic alterations, such as frequent mutations of the ATM-rich interactive domain IA (ARID1A, 40–57%) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA, 33–50%) genes, amplifications of AKT2 (14%) and protein phosphatase, Mg²⁺/Mn²⁺ dependent 1D (PPM1D, 10%) genes, and loss of mismatch repair genes (7–18%) (Wilson and Roberts, 2011; Tan et al., 2013; Itamochi et al., 2015; Friedlander et al., 2016). Loss of phosphatase and tensin homolog (PTEN, 40–51%) expression and amplification and overexpression of Erb-b2 receptor tyrosine kinase 2 (ERBB2, 9.3–14%) has also been reported (Tan et al., 2013; Friedlander et al., 2016). Most of these studies have analysed only targeted genes, although ovarian cancers, including OCCC, have heterogeneous gene alterations (Bast et al., 2009; Tan et al., 2011).

Recently, high-throughput sequencing of DNA has been successfully applied to several cancers, enabling the discovery of cancer genes and network-attacking mutations that can possibly translate into advances in cancer diagnosis and treatment (Garraway and Lander, 2013; Creixell translate into advances in cancer diagnosis and treatment.

Clinical samples. Ovarian tumour tissue samples and matched blood samples were obtained from 55 Japanese women diagnosed with OCCC and treated surgically at Iwate Medical University, Tottori University, Niigata University, Tokai University, Tohoku University, Juntendo University, Shikoku Cancer Center, Kurume University, Hokkaido University, Keio University, Osaka University, Kagoshima City, or Hirosaki University Hospitals from 2003 to 2012. The study protocol was approved by the institutional review boards of each institution and all patients submitted written informed consent before collection of specimens, in accordance with institutional guidelines. These patients underwent complete surgical staging, including intraperitoneal cytology, bilateral salpingo-oophorectomy, hysterectomy, omentectomy, pelvic-/paraortic lymphadenectomy, and aggressive cytoreductive surgery, followed by platinum-based chemotherapy. Thirty-five (64%) patients received postoperative chemotherapy, consisting of paclitaxel at 175 mg·m⁻² plus an area under the curve of 6.0 mg·ml⁻¹ of carboplatin on day 1, every 3 weeks for up to six cycles. The remaining 20 (36%) patients received 60 mg·m⁻² of irinotecan on days 1, 8, and 15, plus 60 mg·m⁻² of cisplatin on day 1, every 4 weeks for up to six cycles. There were 28 patients with stage I disease, 5 with stage II, 19 with stage III, and 3 with stage IV according to the surgicopathological staging guidelines of the International Federation of Gynaecology and Obstetrics (FIGO).

Tumour tissue samples (~1 g each) were collected during surgery and then immediately frozen in liquid nitrogen and stored at −80°C until assayed. Blood was withdrawn and fractionated as a pretreatment to separate the buffy coat, comprising white blood cells and platelets, from erythrocytes and plasma. The buffy coat was cryopreserved and was used for normal genomic DNA matching to the tumour samples. Before whole-exome sequencing, germline single-nucleotide polymorphism analysis was performed on tumour-blood pairs to confirm identities, showing matching of all tumour samples to the respective blood samples.

Massively parallel sequencing. Genomic DNA from tumour and blood was extracted using the DNeasy Blood & Tissue DNA kit (QIAGEN, Hilden, Germany). Exome capture was performed using the Agilent SureSelect BTA XT AUTO Human All Exon V5 + IncRNA Platform (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. Whole-genome sequencing was performed on the Illumina HiSeq platform (Illumina, Inc., San Diego, CA, USA) according to standard protocols (Imielinski et al., 2012). Base calling and quality scoring was conducted using Real-Time Analysis Software, then de-multiplexing and generation of FASTQ files were performed using the CASAVA package provided by Illumina (Illumina, Inc.). The data set analysed here (the data set ID: JGAS000000000076) is available at the National Bioscience Database Center (NBDC) website (https://humanbdb.bioscience-dbcp.jp/en/hum0067-v1) in controlled access.

Detection of somatic single-nucleotide variants (SNVs) and short insertion and deletion (INDEL) variants. Mapping of genomes of raw sequence data (FASTQ files) was performed using the Burrows–Wheeler alignment algorithm (Li and Durbin, 2009). Then, realignment, recalibration, and generation of binary sequence alignment/map files were performed using the GATK toolkit and SAMtools (Li et al., 2009; McKenna et al., 2010). The SNVs and INDELS were detected by comparing sequence data from tumour-blood pairs using the MuTect, VarScan, and SomaticIndelDetector GATK algorithms (Koboldt et al., 2012; Cibulskis et al., 2013). Then, the ANNOVAR tool was used to annotate all SNVs and INDELS (Wang et al., 2010).

Somatic copy number variation (CNV) detection. Exome CNV and Control-FREEC were used to analyse the copy number (CN) in sequence data from tumour-blood pairs (Sathirapongsasuti et al., 2011; Boeva et al., 2012). A CN of ≥4 was considered as a CN gain and the size of regions of ≤3 Mb were defined as focal amplifications.

Statistical analyses. Statistical analyses were performed with JMP, version 12, software (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism, version 7, software (GraphPad Software, Inc., La Jolla, CA, USA). The χ² test, Fisher’s exact test, and unpaired t-test were used for statistical analysis. Cluster analyses were performed by hierarchical clustering with Ward’s minimum
variance method. Survival distributions were calculated using the Kaplan–Meier method and the significance of apparent differences in survival distribution between groups was tested using the log-rank test. In addition, the Cox proportional hazards model for multivariable analysis was applied. A probability (P) value of <0.05 was considered statistically significant.

RESULTS

Identification of gene mutations. A total of 4792 genomic alterations were identified in the 55 tumours with a median of 62 alterations per tumour (range 25–2676), although no germline mutations and CNVs were correlated with these genomic alterations. Of these, 37 genes were found to be mutated in at least 10 (18%) of the tumours, with the most common alterations being NBPF members 20 (67% of tumours), 10 (60%), and 14 (60%) (Supplementary Table 1). A total of 51 (93%) cases had a mutation in NBPF20, 10, and 14 genes, and these mutations were all nonsynonymous SNVs. The relationship between nonsynonymous SNVs of these genes and clinicopathological factors and prognostic significance in several cancers has been reported recently, it is unclear whether gene alterations affect the clinical behaviour of OCCC. Therefore, we investigated whether the alteration status could be a prognostic biomarker for this disease.

Pathways influencing patient’s outcome. Although the relationship between molecular alterations of genes and clinicopathological variables and prognostic significance in several cancers has been reported recently, it is unclear whether gene alterations affect the clinical behaviour of OCCC. Therefore, we investigated whether the alteration status could be a prognostic biomarker for this disease.

Somatic mutations of ARID1A, which encodes a member of the switch/sucrose non-fermentable (SWI/SNF) family protein, were identified in approximately half of OCCC cases (Wilson and Roberts, 2011; Itamochi et al, 2015). We therefore examined mutations of the SWI/SNF subunit genes. A total of 28 (51%) cases had a mutation in ARID1A (42%), ARID1B (18%), B-cell CLL/Lymphoma 11A (BCL11A) (2%), double PHD fingers 1 (DPF1) (2%), SWI/SNF related, matrix associated, actin dependent regulator of chromatin (SMARC) A1 (2%), SMARC A2 (2%), SMARC A4 (5%), or SMARCC1 (2%) (Figure 2A). These mutations occurred more frequently in patients with FIGO stage I/II disease.
compared with those with stage III/IV (Table 1). Univariate analysis demonstrated that mutations of the SWI/SNF subunit genes tend to correlate with better OS among patients with OCCC, with 3-year survival rates of 91% and 53% (hazard ratio 0.11 (95% CI 0.01–0.54), P = 0.0249), respectively (Figure 3C). After adjustment for patient age, FIGO stage, and treatment regimen, activation of this pathway was also correlated with better OS of patients with OCCC (hazard ratio 0.27 (95% CI 0.06–0.86), P = 0.0276), respectively. Interestingly, the OS rate was also significantly higher in patients with FIGO stage I or II OCCC with activated pathway compared with not activated, with 3-year survival rates of 94% and 61% (hazard ratio 0.19 (95% CI 0.04–0.83), P = 0.0276), respectively.

Altering of the RTK/Ras signalling pathways genes were found in a total of 16 (29%) cases, including amplifications of ERBB2 (11%) and ERBB3 (5%), and mutations of ERBB2 (4%), ERBB3 (7%), KRAS (9%), and BRAF (2%) (Figure 4A and B). Of these alterations, the RTK/Ras signalling pathway was activated in 11 (20%) cases. Univariate analysis revealed that activation of this signalling pathway was correlated with better OS of patients with OCCC, with 3-year survival rates of 91% and 53% (hazard ratio 0.35 (95% CI 0.13–0.94), P = 0.0373), respectively (Figure 4C). After adjustment for patient age, FIGO stage, and treatment regimen, activation of this pathway was also correlated with better OS of patients with OCCC (hazard ratio 0.11 (95% CI 0.01–0.54), P = 0.0034).

Multivariable analysis of age, FIGO stage, and genes alterations was performed that found that FIGO stage, activation of PI3K/Akt

Table 1. Comparison of clinicopathological factors between gene alterations in ovarian clear cell carcinoma

| SWI/SNF mutation | PI3K/Akt activation | RTK/RAS activation |
|------------------|---------------------|---------------------|
| **Yes** | **No** | **P-value** | **Yes** | **No** | **P-value** | **Yes** | **No** | **P-value** |
| Age (years) | | | | | | | | | |
| Range | 36–67 | 36–68 | 0.7261 | 36–67 | 36–86 | 0.2981 | 46–68 | 36–86 | 0.5253 |
| Median | 56 | 35.5 | 55 | 50 | 55.5 | | | | |
| FIGO stage | | | | | | | | | |
| I | 17 | 14 | 0.0208* | 14 | 14 | | 7 | 21 | >0.9999* |
| II | 4 | 3 | 0.0493* | 3 | 2 | | 0 | 5 | |
| III | 5 | 14 | | 5 | 3 | | | | |
| IV | 1 | 3 | | 0 | 0 | | | | |
| Regimens | | | | | | | | | |
| TC | 13 | 8 | 0.0111 | 27 | 6 | 0.0013 | 26 | 18 | 0.2932 |
| CPT-P | 15 | 14 | | 5 | 3 | | | | |
| Deaths | 8 | 3 | 0.1022 | 19 | 19 | 0.0017 | 1 | 21 | 0.0356 |

Abbreviations: CPT-P = irinotecan (60 mg m⁻²) on days 1, 8, and 15 + cisplatin (60 mg m⁻²) on day 1, every 4 weeks; FIGO = International Federation of Gynecology and Obstetrics; PI3K = phosphatidylinositol-3-kinase; RTK = receptor tyrosine kinase; SWI/SNF = switch/sucrose non-fermentable; TC = paclitaxel (175 mg m⁻²) + carboplatin (an area under the curve 60 mg ml⁻¹) on day 1, every 3 weeks.

*FIGO stage I vs III/IV.
subunit of the SWI/SNF complex (Wilsker et al, 2004; Wilson and Roberts, 2011). These observations suggest that one of these mutations may be sufficient for deterioration of the tumour suppressor role of this complex in OCCC.

The impact of ARID1A status on the treatment outcome of patients with OCCC has been evaluated. Several studies failed to find a correlation between negative expression of the ARID1A protein or mutations of the gene and OS of patients with OCCC (Itamochi et al, 2012; Zang et al, 2013). However, we previously found that the 5-year OS rate for FIGO stage I or II OCCC patients with negative tumour expression of ARID1A was lower than that of patients with positive tumour expression of ARID1A (74% vs 91% respectively). Another study showed that the loss of ARID1A expression was significantly correlated with shorter progression-free survival for patients with OCCC, but not OS (Katagiri et al, 2012). In contrast, Abou-Taleb et al (2016) reported that the loss of expression of one or multiple SWI/SNF subunit proteins demonstrated aggressive behaviour and poor prognosis of OCCC. In this study however, we observed no significant difference in OS between patients with mutations of the SWI/SNF subunit genes and those with the wild-type phenotypes of these genes, regardless of FIGO stage. Therefore, further studies are needed to elucidate the prognostic significance of SWI/SNF subunit alterations in OCCC.

The relationship between the loss of ARID1A expression and activation of the PI3K/Akt pathway has been reported in various cancers, including OCCC, suggesting a collaboration in tumourigenesis (Yamamoto et al, 2012; Zang et al, 2013; Bosse et al, 2013; Huang et al, 2014). Yamamoto et al (2012) reported that mutations of PIK3CA (which encodes the catalytic subunit p110α of PI3K) were detected in 40% (17/42) of OCCC tumours and a majority (71%) of these were found in ARID1A-deficient carcinomas. Another study also showed that the loss of ARID1A was more frequent in OCCC tumours with an activated PI3K/Akt pathway.
(PIK3CA mutations or loss of PTEN expression) (54%) than those without alterations to the PI3K/Akt pathway (30%) (Huang et al, 2014). Indeed, in the present study, 82% of tumours with activation of the PI3K/Akt pathway were observed in tumours with mutations of the SWI/SNF subunit genes. Interestingly, clustering analysis revealed that tumours in cluster 2 were almost lacking mutations of ARID1A, PIK3CA, and ARID1B, and patients in this cluster tended to have worse OS compared with those in clusters 1 and 3. Furthermore, univariate and multivariable analyses revealed that activation of the PI3K/Akt signalling pathway, but not mutations of the SWI/SNF subunit genes, was correlated with better OS of patients with OCCC. Several studies have shown that PIK3CA mutations or overexpression were correlated with improved OS of patients with OCCC (Rahman et al, 2012; Abe et al, 2013). Other studies, however, reported that PIK3CA status was not a prognostic factor for these patients (Huang et al, 2014; Ye et al, 2016). Although the prognostic significance of PIK3CA alterations remains controversial, these novel findings suggest that activation of the PI3K/Akt signalling pathway may have some effect on the treatment outcome of patients with OCCC.

Amplification and overexpression of ERBB2, also known as human epidermal growth factor receptor 2 (HER2), and mutations of KRAS have been reported in 9.3–14% and 4.7–13% of OCCC cases respectively (Tan et al, 2011, 2013; Friedlander et al, 2016; Zannoni et al, 2016). Overexpression of HER2 is associated with poor sensitivity to conventional anticancer agents and poor prognosis in several types of tumours, including ovarian cancer (Kim et al, 1998; Rolitsky et al, 1999; Itamochi et al, 2008). On the contrary, Nodin et al (2013) reported that a KRAS mutation was associated with a significant improvement in cancer-specific survival in patients with ovarian endometrioid carcinoma. However, the prognostic significance of these signalling pathways for OCCC remain largely unknown. Here, we found that the activation of the RTK/Ras signalling pathway was observed in 20% of tumours and was a favourable prognostic factor for OCCC. Moreover, the RTK/Ras pathway and its downstream signalling pathway are thought to be potential targets for cancer therapy (Mandal et al, 2016). We previously reported that inhibition of mitogen-activated protein kinase (MEK) 1/2, which is the downstream signalling cascade of RTK/Ras, by selumetinib reduced growth of OCCC cell lines and suppressed tumour growth in a OCCC xenograft model (Bartholomeusz et al, 2012). These findings suggest that the RTK/Ras signalling pathway may be an important prognostic biomarker for patients with OCCC and a potential therapeutic target for OCCC.

The genomic alterations have also been examined in high-grade serous ovarian cancer (HGS-OvCa), which is the most common...
histological subtype, by the Cancer Genome Atlas (TCGA) study (Cancer Genome Atlas Research Network, 2011). These TCGA analyses reveal that the BRCA1 and BRCA2 mutations in 20% HGS-OvCa samples triggered aberrations in DNA damage repair by homologous recombination. Patients with germline BRCA1/2 mutation are thought to have clinical benefit from using PARP (poly ADP-ribose polymerase) inhibitors, such as olaparib (Sato and Itamochi, 2015). However, in this study of OCCC, only 3 cases (5%) had somatic BRCA2 mutations (2 nonsynonymous SNV and 1 frameshift insertion) (data not shown). Similarly, almost all HGS-OvCa tumours (96%) had mutations in TP53, but 3 OCCC tumours (5%) had mutations (1 stopgain, 1 frameshift deletion, and 1 nonsynonymous SNV) in this gene (data not shown). On the contrary, ARID1A and PIK3CA mutations were more common in OCCC cases compared with that in HGS-OvCa. These differences between ovarian cancer subtypes suggested that subtype-specific treatment strategies might be needed to improve ovarian cancer outcomes.

The limitations of this study are that we have analysed only DNA sequencing data in a relatively small number of OCCC samples, and it lacks independent validation of the prognostic association. Therefore, the sequencing of larger OCCC cohorts will be necessary to determine a more comprehensive genomic landscape for OCCC. Furthermore, precise prevalence frequency and prognostic significance for the mutated genes detected in our analysis also need to be validated by DNA sequencing as well as by multiple-assay platform. Recently, Friedlander et al (2016) examined the results of a multiplatform profiling panel, such as DNA sequencing, immunohistochemistry, fluorescent or chromogenic in situ hybridisation, and RNA fragment analysis, in OCCC to identify the potential therapeutic targets. Consistent with their results, we confirmed that the PIK3CA/ Akt/mTOR pathway was altered in 61% OCCC tumours.

In summary, our data showed that mutations of ARID1A and PIK3CA were frequently observed in OCCC tumours. We also found that the activation of PI3K/Akt and RTK/Ras signalling pathways may be a favourable prognostic marker for patients with OCCC. We believe that these whole-genome sequencing data will be valuable and useful for further analysis of the molecular and biological characteristics of OCCC, and may lead to the establishment of novel treatment strategies to improve survival of patients with OCCC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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