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Exome Sequencing Landscape Analysis in Ovarian Clear Cell Carcinoma Shed Light on Key Chromosomal Regions and Mutation Gene Networks

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Previous studies have reported genome-wide mutation profile analyses in ovarian clear cell carcinomas (OCCCs). This study aims to identify specific novel molecular alterations by combined analyses of somatic mutation and copy number variation. We performed whole exome sequencing of 39 OCCC samples with 16 matching blood tissue samples. Four hundred twenty-six genes had recurrent somatic mutations. Among the 39 samples, *ARID1A* (62%) and *PIK3CA* (51%) were frequently mutated, as were genes such as *KRAS* (10%), *PPP2R1A* (10%), and *PTEN* (5%), that have been reported in previous OCCC studies. We also detected mutations in *MLL3* (15%), *ARID1B* (10%), and *PIK3R1* (8%), which are associations not previously reported. Gene interaction analysis and functional assessment revealed that mutated genes were clustered into groups pertaining to chromatin remodeling, cell proliferation, DNA repair and cell cycle checkpointing, and cytoskeletal organization. Copy number variation analysis identified frequent amplification in chr8q (64%), chr20q (54%), and chr17q (46%) loci as well as deletion in chr19p (41%), chr13q (28%), chr9q (21%), and chr18q (21%) loci. Integration of the analyses uncovered that frequently mutated or amplified/deleted genes were involved in the *KRAS*/phosphatidylinositol 3-kinase (82%) and *MYC*/retinoblastoma (75%) pathways as well as the critical chromatin remodeling complex switch/sucrose nonfermentable (85%). The individual and integrated analyses contribute details about the OCCC genomic landscape, which could lead to enhanced diagnostics and therapeutic options. (*Am J Pathol* 2017, 187: 2246–2258; <http://dx.doi.org/10.1016/j.ajpath.2017.06.012>)

Ovarian cancer is the poorest prognostic disease in gynecologic malignant tumors and is complicated by the fact that the prognoses and corresponding behaviors in histologic subtypes are distinct.¹ High-grade serous ovarian cancer is the most frequent subtype, accounting for 68% of ovarian cancer.¹ The ovarian clear cell carcinoma (OCCC) subtype is highly resistant to chemotherapy and often associated with endometriosis.² The OCCC subtype accounts for approximately 4% to 12% of all epithelial ovarian carcinomas in European and North American countries, whereas its prevalence increases to 15% to 20% in Japan.^{3,4} To date, only eight OCCC samples have been analyzed by exome sequencing,⁵ which is an extremely small sample size compared with the 316 high-grade serous ovarian cancer

samples analyzed by The Cancer Genome Atlas.⁶ Hence, the genomic landscape of the rarer OCCC subtype remains insufficiently investigated on a larger scale.

Thus far, OCCC-specific somatic mutations in focused target-level studies of *ARID1A*,^{5,7} *PIK3CA*,^{5,8,9} and *PPP2R1A*^{5,10} and copy number amplification of *ZNF217*^{11,12} have been reported in Caucasian populations. Interestingly, these genetic alterations of the OCCC subtype are entirely different from the high-grade serous ovarian

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cancer subtype in which *TP53* and *BRCA* alterations have been observed frequently.¹³

A pan-cancer study performed mutation and copy number analyses using large numbers of samples of various tumor types, where analyses grouped tumor types exclusively by mutation or copy number alteration.¹⁴ OCCC was not specifically analyzed as a tumor type. However, it is particularly interesting because suppression of a single tumor growth signaling mechanism via molecularly targeted chemotherapy leads to increased signaling in a different mechanism,^{15,16} and hence analysis of only mutations or only copy number alterations alone is insufficient. A thorough investigation of both independent mutation and copy number analyses as well as a joint, integrated analysis for OCCC that clarifies the separate and joint landscapes has yet to be reported. Therefore, we aim to clarify the patterns of genomic alterations in OCCC and to identify whether such alterations are caused by mutation addiction, copy number addiction, or both.

Until recently, single-nucleotide polymorphism (SNP) array technology has been used for differential analysis of patients, and it is still a cost-effective competing technology to newer next-generation sequencing.¹⁷ In the case of OCCC, samples and data are highly limited; standard regions and hotspots available in SNP arrays may not sufficiently cover the subtype-specific alterations that explain the disease. The resolution of whole exome sequencing circumvents these shortcomings and is applicable for the detection of novel OCCC-specific mutation elements.

In this study, we applied whole-exome sequencing to 39 OCCC samples, the largest OCCC-specific collection to our knowledge, and to 16 noncancerous blood tissue samples provided by a subset of the same patients. To elucidate altered genetic elements, we compared the tumor-blood sequencing results and additionally profiled the tumor samples against a public database of 1208 healthy Japanese exomes.¹⁸ In addition, copy number analysis of these OCCC exome sequencing data sets was undertaken. Critically, we integrated the independent single-nucleotide variant (SNV) and copy number variant (CNV) analyses, aiming to improve the resolution of the molecular profiling of OCCC, such as identifying the links between functional sub-networks of genes with molecular alterations.

Materials and Methods

Samples

Ovarian tumor samples were collected from 39 patients with OCCC who had no pretreatments and underwent surgery between 1997 and 2013 at Kyoto University Hospital. This study was approved by an institutional ethics committee in which donors gave written informed consent in accordance with institutional and national guidelines. DNA was extracted from the 39 frozen tumor tissues stored at -80°C and from matching blood lymphocyte samples available

from 16 of the 39 patients. During the initial years of sample collection, it was not assumed that samples were going to be used for exome comparison, and consequently, blood lymphocyte samples could be not extracted from 23 of the 39 patients because of a lack of patient consent. The genomic DNAs were purified using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. Quality of DNA was assessed by the DNA concentration ($>100\text{ ng}/\mu\text{L}$) and electrophoresis. In total, 3 μg of genomic DNA was used for exome sequencing.

Exome Sequencing

Whole exome sequencing was performed using Agilent SureSelect Human All Exon V5 + UTRs (Agilent Technologies, Santa Clara, CA). Exome sequencing data were generated by 100-base paired-end reads on an Illumina HiSeq 2000 system (Illumina Inc., San Diego, CA) and analyzed through an in-house pipeline. Paired-end reads in FASTQ files were mapped on to the human reference genome sequence (GCRh37/hg19) with Burrows-Wheeler Aligner (BWA) software version 0.7.10 (<http://bio-bwa.sourceforge.net>).¹⁹ The output files in SAM format were converted into sorted BAM files, and duplicate reads were removed using SAMtools version 1.0 (<http://samtools.sourceforge.net>).²⁰ Downstream steps, including insertion/deletion realignment, recalibration of base quality, variant quality score recalibration, and variant calling, were performed with the Genome Analysis Toolkit version 3.2.²¹ ANNOVAR version 2014Jul14²² was used to annotate variants in variant call format files. Mean coverage of target regions was $92.6\times$ (range, $68.8\times$ to $122.7\times$), and a mean of 92% of target regions were covered with at least $20\times$ coverage (range, 87% to 96%). A total of 4620 variants were left for subsequent analyses after filtering out variants with quality scores <40 or with sample call rates $<95\%$ (52 of 55 samples) to avoid misidentification of mutations occurring in OCCC samples. Samples were further filtered as explained below (Figure 1).

Sanger Sequencing

Mutations in specific genes were confirmed by Sanger sequencing (Table 1). Ten nanograms of genomic DNA was mixed in a total 50- μL reaction that contained 25 μL of Emerald Amp MAX PCR Master Mix (Takara Bio Inc., Kusatsu, Shiga, Japan), 1 μL (100 pmol/ μL) of forward primer, and 1 μL (100 pmol/ μL) of reverse primer in a PCR tube. PCR amplification was performed using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) by the following procedure: denaturation at 98°C for 5 minutes; 30 cycles of the following three per-cycle conditions of 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; subsequent holding at 72°C for 1 minute; and finally incubation at 12°C . The primer specificity and optimal cycling conditions were verified by detecting single-band amplicons of the PCR products. The PCR products were purified with

Figure 1 Workflow for detection of somatically mutated genes and copy number alterations in ovarian clear cell carcinoma (OCCC) samples. An initial pool of somatic mutations was obtained from exome sequencing data of tumors by comparing with their normal counterparts. These mutations were further refined by using functional annotation and variants found in public and private exome sequencing data from healthy individuals. A list of genes that had at least two remaining somatic mutations after filtering was intersected with known cancer driver genes to extract reliable OCCC-associated genes. For copy number analysis, median coverages of each exon were compared between tumor and normal tissues. For the samples without normal counterparts, the mean coverage from the other 16 patients was used as a surrogate. Copy number alterations were assessed at peak regions, at the gene level, at the arm level, and for genome-wide correlations. CGH, comparative genomic hybridization; CNV, copy number variation; DAVID, Database for Annotation, Visualization and Integrated Discovery; GATK, Genome Analysis Toolkit; PBMC, peripheral blood mononuclear cell; PPI, protein-protein interaction.

QIAquick PCR Purification Kit (Qiagen), and the 0.3- μ L DNA template (10 ng) with 3.2 μ L of primer (100 pmol/ μ L) was run on the 3130xl Genetic Analyzer (Thermo Fisher Scientific/Applied Biosystems, Waltham, MA).

Somatic Mutation Gene Analysis

Functional mutations [ie, missense, truncation (nonsense variants and indels in coding regions), and splicing variants] were extracted after filtering out the variants found in exome sequence data sets from at least one of the following: normal tissue counterparts of the 16 patients with OCCC and/or 1208 healthy Japanese individuals in the Human Genetic Variation Database version 1.0 (<http://www.hgvd.genome.med.kyoto-u.ac.jp>) or 338

cancer-free Japanese individuals in an in-house database. This application of multiple criteria serves as a quality control check and a method to incorporate as many tumors as possible into the analysis, even if matched normal samples are not available for reasons mentioned above. Therefore, although strict tumor-normal matched pair criteria for the definition of somatic mutation cannot be applied, our quality controls provide at least a basis for rationalizing that the mutations are likely so. Along this line, the mutations detected were qualified as somatic.

A list totaling 565 unique known driver genes was obtained from the IntOGen Mutational Cancer Driver Genes database release 2012.12^{23,24} (<https://www.intogen.org>), and the Pan-cancer study.²⁵

MutSigCV version 1.4²⁶ was applied for detecting genes that had functional somatic mutations more frequently than statistical expectation in the 39 OCCC tumor samples.

MutationMapper version 1.0.1 developed by cBioPortal (Memorial Sloan Kettering Cancer Center, New York, NY; <http://www.cbioportal.org/tools.jsp>)²⁷ was used for visualization of distribution patterns of the somatic mutations identified in *PIK3CA* and *ARID1A*.

Copy Number Analysis

Median coverages of each exon were calculated, and those from tumor and normal tissues were compared for copy number analysis. To analyze the 23 samples for which corresponding normal tissues were not available, the mean coverage of the exome sequencing data from normal tissues of the other 16 patients was used as a surrogate coverage value. Copy number value was generated with transformed $\log_2$ ratios of tumor/normal coverages after applying a moving mean of 60 exons. As a validation set, an array comparative genomic hybridization (CGH) data set obtained from 117 OCCC cases with $\log_2$ normalized data (GSE58342)²⁸ was downloaded from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE58342). Five array CGH samples were excluded from the analysis because of duplication. Bioconductor package copynumber,²⁹ an extension to R software version 3.1.2 (The R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org>), was used for analyses and visualization of CNVs, where CNV was determined by the $\log_2$ ratio having a value of >0.25 for amplification and <-0.25 for deletion in a single segmentation analysis.^{28,30–32}

GISTIC version 2.0 (The Broad Institute, Boston, MA; <http://archive.broadinstitute.org/cancer/cga/gistic>) analysis was performed for identifying copy number amplifications and deletions at the arm level, as well as at peak loci and gene levels in the two data sets.³³ A threshold for variation was set to 0.25 (Kyoto) or 0.15 (GSE58342) of the absolute copy number $\log_2$ scaled values. The correlations of peak and arm-level amplifications and deletions found in the two data sets were closely examined.

Pathway Analysis

Protein-protein interaction network analysis of genes retained after somatic mutation filtering (Figure 1) was performed using STRING version 10 (<http://www.string-db.org>).³⁴

Candidate somatic mutations were mapped onto a protein-protein interaction network. Networks of less than five interactions were excluded. Network visualization was performed in Cytoscape software version 2 (<http://www.cytoscape.org>).³⁵ Using genes in the extracted networks, pathway analyses with the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources version 6.7 (NIH, Bethesda, MD; <https://david.ncifcrf.gov>) were performed to annotate genes with functional roles. Nodes of the two networks were colored for functional roles by using the MultiColoredNodes plugin within Cytoscape.³⁶

Integrated analysis of SNVs and CNVs was performed by focusing on the pathways enriched with genes found to be frequently altered in our analyses.

Quality Assessment for Somatic Mutation Genes

For evaluating the quality of mutation data from the cases with or without matched normal samples, the Fisher exact test was used for counts of variants between 16 paired cases and 23 unpaired cases using 426 candidate somatic mutation genes. Then we compared core somatic mutations listed in 42 IntOGen pan cancer genes and the 46 protein-protein interaction (PPI) network genes in each of the subgroups. Moreover, we examined variant allele frequency (VAF) as calculated by the variant allele percentage and total read counts, which represent copy number status, in truncating and missense mutations. Variants with >30 total reads were retained. The truncation mutation variants ($n = 172$) and missense mutation variants ($n = 854$) were examined. The most frequently mutated gene, *ARID1A* in truncating mutations, and the gene with the highest frequency of missense mutations, *PIK3CA*, were visualized by scatterplot coloring for identification.

Immunohistochemical Analysis for BAF250a Expression

Immunohistochemical staining for BAF250a was performed and evaluated similarly to methods previously reported.³⁷ Briefly, protein expression was evaluated by determining the Immuno-Reactive Score (IRS) ranging from 0 to 300, which is obtained by multiplying the percentage of positively stained cells by the intensity of the reaction, which can take the values 0, 1, 2, or 3. Scores between 0 and 49 were classified as negative, whereas scores ≥ 50 were considered to indicate positive expression. The association between BAF250a expression and *ARID1A* mutation status with or without *ARID1A* deletion was examined.

Immunohistochemical Analysis for PTEN Expression

PTEN immunohistochemistry was performed with the PTEN monoclonal mouse antibody (clone 6H2.1; Dako Cytomation, Carpinteria, CA). Antigen retrieval was accomplished using EDTA buffer (pH 9.0) with steaming for 15 minutes, followed by the PTEN antibody (dilution 1:150). Diaminobenzidine was used as the chromogen. For tumors with mutant PTEN and loss of PTEN protein expression, positively stained blood vessels and stromal cells were used as an internal positive control. An IRS ranged from 0 to 300, similar to BAF250a described above, with an identical cutoff of 50.

Comparison of Copy Number by Exome Sequencing versus SNP Array

For the subset of 12 OCCC samples with matching SNP arrays downloaded from Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE30311),³⁰ copy number analysis was performed using Bioconductor packages *cn.farms*³⁸ and *copynumber*.²⁹

Statistical Analysis

R version 3.1.2 was used for statistical analyses. The Fisher exact test, *t*-test, and Pearson correlation test were used where appropriate. *P* values were adjusted to false discovery rate (FDR) *q*-values using the *p.adjust* function in R with parameter method = *fdr*.

Results

Identification of Somatic Mutations in OCCC

In total, 92,130 variants were identified, containing 3228 functional variants after variants found in matching blood samples or in the aforementioned healthy exomes were filtered out; 2838 nonsynonymous (88%), 279 truncation [143 stop gain/loss (5%) and 136 in-/out-of-frame insertion/deletion (4%)], 66 nonframeshift insertion/deletion (2%), and 45 splicing variants (1%) were located in 2691 genes (Figure 1). Among a total of 3007 nonindel variants, C to T, T to C, C to G, C to A, T to G, and T to A transitions accounted for 50%, 16%, 15%, 10%, 5%, and 4% of changes, respectively (Figure 2, A and B).

A total of 426 genes had at least two somatic variants (Supplemental Table S1) in which 42 genes were included in a prominent list of cancer driver genes previously reported.^{23–25} Nine of those 42 genes were mutated in >10% of 39 samples, of which *ARID1A* had the highest number of mutations (29 truncation and 1 missense) (Figure 2C). MutSigCV^{26,39} identified *ARID1A* and *PIK3CA* as significantly mutated genes in the tumor samples ($q < 0.0001$). Twenty-four (62%) and 20 (51%) of 39 samples had respective mutations in *ARID1A* or *PIK3CA*

Table 1 Summary of Primers for Sanger Sequencing

Chromosome	Position	Gene	Forward primer
1	27056213	<i>ARID1A</i>	5'-ATTCAGTGGCCAGAGGCCAT-3'
1	27056232	<i>ARID1A</i>	5'-ATTCAGTGGCCAGAGGCCAT-3'
1	27056319	<i>ARID1A</i>	5'-ATTCAGTGGCCAGAGGCCAT-3'
1	27057727	<i>ARID1A</i>	5'-ACTCATCATCAGTGCATAGC-3'
1	27057818	<i>ARID1A</i>	5'-ACTCATCATCAGTGCATAGC-3'
1	27058063	<i>ARID1A</i>	5'-ACTCATCATCAGTGCATAGC-3'
1	27058070	<i>ARID1A</i>	5'-ACTCATCATCAGTGCATAGC-3'
1	27087896	<i>ARID1A</i>	5'-CTGTTGGCTGGATCTCTTTG-3'
1	27092737	<i>ARID1A</i>	5'-AGTTCTAGTTTTGGGGACCC-3'
1	27092787	<i>ARID1A</i>	5'-AGTTCTAGTTTTGGGGACCC-3'
1	27093048	<i>ARID1A</i>	5'-AGTTCTAGTTTTGGGGACCC-3'
1	27099102	<i>ARID1A</i>	5'-CGTGTGTTTGTGTGAGAGTT-3'
1	27099325	<i>ARID1A</i>	5'-CGTGTGTTTGTGTGAGAGTT-3'
1	27099864	<i>ARID1A</i>	5'-GAGATCAAACCTGAACCTG-3'
1	27100139	<i>ARID1A</i>	5'-GAGATCAAACCTGAACCTG-3'
1	27100291	<i>ARID1A</i>	5'-GAGATCAAACCTGAACCTG-3'
1	27100953	<i>ARID1A</i>	5'-CCTGAGTGCAGAGTATTAAC-3'
1	27101092	<i>ARID1A</i>	5'-CTTCCCCTCAGCAAGATGTAT-3'
1	27101099	<i>ARID1A</i>	5'-CTTCCCCTCAGCAAGATGTAT-3'
1	27101273	<i>ARID1A</i>	5'-CTTCCCCTCAGCAAGATGTAT-3'
1	27102196	<i>ARID1A</i>	5'-GACAGACAGCTTAGAAGCCA-3'
1	27105553	<i>ARID1A</i>	5'-TGCTCTGTGGAGAACCTTTG-3'
1	27106354	<i>ARID1A</i>	5'-AGAGTTCAGAGGCCATCAAG-3'
1	27106478	<i>ARID1A</i>	5'-AGAGTTCAGAGGCCATCAAG-3'
1	27106861	<i>ARID1A</i>	5'-AGAGTTCAGAGGCCATCAAG-3'
1	27107152	<i>ARID1A</i>	5'-CCGGAACATCAAGATCCTAG-3'
3	178927980	<i>PIK3CA</i>	5'-CTCATGCTTGCTTTGGTTCA-3'
3	178936082	<i>PIK3CA</i>	5'-GATTGGTTCTTTCTGTCTCT-3'
3	178936091	<i>PIK3CA</i>	5'-GATTGGTTCTTTCTGTCTCT-3'
3	178936092	<i>PIK3CA</i>	5'-GATTGGTTCTTTCTGTCTCT-3'
3	178952085	<i>PIK3CA</i>	5'-CAGGAGATGTGTTACAAGGC-3'
10	89653843	<i>PTEN</i>	5'-GCAGGTGTCAATTTTGGGG-3'
10	89692905	<i>PTEN</i>	5'-CCACAGTTGCACAATATCCT-3'
10	89711900	<i>PTEN</i>	5'-CTGTCCACCAGGGAGTAACT-3'
19	52715982	<i>PPP2R1A</i>	5'-TTCATAGGGCTGGGAAGACA-3'
6	157522623	<i>ARID1B</i>	5'-GAAGATGCAGAAGGTCATGC-3'
6	157517352	<i>ARID1B</i>	5'-GACAGAGTGAGACTCCATCT-3'
6	157528310	<i>ARID1B</i>	5'-GTCCGTAGCTTGTCAATTCGT-3'
19	11098500	<i>SMARCA4</i>	5'-AGAGCTCAACATGACGCCCT-3'

(table continues)

genes (Figure 2D). For the key *PPP2R1A* gene previously analyzed in ovarian and endometrial cancers,¹⁰ four of 39 samples (10%) contained the p.R183W hot spot mutation. Chromatin modification–related *KMT2C/MLL3* and chromatin remodeling-related *ARID1B* were identified for novel association with OCCC, having mutation rates of 15% and 10%, respectively. We were also able to identify *KRAS*, *PIK3R1*, and *PTEN* with considerable mutation frequencies of 10%, 8%, and 5%, respectively (Figure 2, C and D).

We further examined mutation patterns of the frequently mutated genes *ARID1A* and *PIK3CA*. Across 20 cases of mutated *PIK3CA*, five missense mutations were identified, in which the p.H1047R hot spot mutation was observed in 11 cases (Figure 2E). In contrast, 29 of 30 mutations identified in *ARID1A* across 24 cases were truncating mutations.

The truncations were found dispersed throughout *ARID1A* (Figure 2F).

A total of 55 of 56 somatic mutations (98%) were validated by Sanger sequencing of *ARID1A* (26/27), *PIK3CA* (19/19), *PPP2R1A* (4/4), *PTEN* (2/2), *ARID1B* (3/3), and *SMARCA4* (1/1) (Table 1).

Identification of CNV and Correlations at Multiple Scales

Averaged CNV signals across the 39 exome sequencing data and across 112 array CGH samples (GSE58342, overlapping OCCC samples excluded) are shown in Figure 3. The overall CNV patterns are highly concordant between these two independent data sets. Here, we focus on

Table 1 (*continued*)

Reverse primer	Verification	Reference sequence	Alternative sequence	Counts
5'-CACAATCACCATCTACCTGC-3'	Verified	G	GC	1
5'-CACAATCACCATCTACCTGC-3'	Verified	G	T	1
5'-CACAATCACCATCTACCTGC-3'	Verified	CAGAGTGCCAT	C	1
5'-TGCTGTGTCGTCGACTGCTG-3'	Verified	C	T	1
5'-TGCTGTGTCGTCGACTGCTG-3'	Verified	CA	C	1
5'-TGCTGTGTCGTCGACTGCTG-3'	Verified	GC	G	1
5'-TGCTGTGTCGTCGACTGCTG-3'	Verified	CCCAGCAG	C	1
5'-GTGTGATACTGGGAGGTACT-3'	Verified	CA	C	1
5'-GGCTTCTTTAGCCCAATGAG-3'	Verified	C	T	1
5'-GGCTTCTTTAGCCCAATGAG-3'	Verified	T	TA	1
5'-AGACTATCTAGTCCGGTGTC-3'	Verified	CA	C	1
5'-CACTGTCATGCCAAGCAAAC-3'	Verified	T	TC	1
5'-CACTGTCATGCCAAGCAAAC-3'	Verified	C	T	2
5'-CCTGAACTCTGAAGAGGGCC-3'	Verified	C	G	1
5'-CCTGAACTCTGAAGAGGGCC-3'	Verified	C	A	1
5'-CCTGCTGTTGCTGTTGATAC-3'	Verified	A	C	1
5'-CTCTGATCTGTGTGCAGCAT-3'	Verified	CTG	C	1
5'-CCTCAGCAAGATGTATACAAC-3'	Verified	G	GT	1
5'-CCTCAGCAAGATGTATACAAC-3'	Verified	C	T	2
5'-CCTCAGCAAGATGTATACAAC-3'	Not detected	C	T	1
5'-CATGGCGGGTAATGATGTCCCTCAA-3'	Verified	C	T	1
5'-CTTGTCCTTGCCTGAAAAGG-3'	Verified	C	T	1
5'-CAGCAGTACCACAGCCATCTC-3'	Verified	C	T	1
5'-CAGCAGTACCACAGCCATCTC-3'	Verified	CT	C	1
5'-CAGCAGTACCACAGCCATCTC-3'	Verified	C	T	2
5'-TCCCACGGCTGTCATGACTG-3'	Verified	G	T	1
5'-AGAGAGAAGGTTTGACTGCC-3'	Verified	T	C	2
5'-GCACTTACCTGTGACTCCAT-3'	Verified	G	A	3
5'-GCACTTACCTGTGACTCCAT-3'	Verified	G	A	3
5'-GCACTTACCTGTGACTCCAT-3'	Verified	A	C	1
5'-GCAATTCCCTATGCAATCGGTC-3'	Verified	A	G,T	11
5'-CAAAGCATTCTTACCTTACTACATC-3'	Verified	G	T	1
5'-TACCTTTTTGTCTCTGGTCC-3'	Verified	G	A	1
5'-GGCTTCTTTAGCCCAATGAG-3'	Verified	G	A	1
5'-CAAAACTCACCTGCTCGTCAG-3'	Verified	C	T	4
5'-TCAGATTAGCAGCCTCCAAC-3'	Verified	G	C	1
5'-GGTCGTAACCTGGCTCCATAG-3'	Verified	A	AT	1
5'-AAGCAGACAAGTCTAGCTGC-3'	Verified	G	A	1
5'-GGAGAAAGTTGAGTGGCTTC-3'	Verified	G	A	1

the whole exome samples. Frequent amplifications on chr8q, including *MYC*; on chr20q, including *ZNF217*; and on chr17q, including *ERBB2*, *HNF1beta*, and *STAT3*, were observed in 64%, 54%, and 46% of 39 samples, respectively. Other loci, including *PIK3CA*, *KRAS*, *CCNE1*, *IL6*, and *IL6R* were also amplified (26%, 18%, 26%, 21%, and 21% of samples, respectively). On the other hand, frequent deletions were observed on chr19p, including *SMARCA4* (41%); on chr13q, including *RB1* (28%); on chr18q, including *SMAD4* (21%); and on chr9q, including *NOTCH1* (21%); the four loci that contained *ARID1A*, *ARID1B*, *SMARCA2*, and *SMARCA4*, which are components of the switch/sucrose nonfermentable (SWI/SNF) complex, were jointly deleted.⁷ Deletions were also found on chr17p where *TP53* is located (Figure 3).

At the chromosome-arm level of resolution, amplified loci were found in both OCCC samples and GSE58342 at 1q, 3q, 7p, 8p/q, 12p, 17q, 19q, and 20p/q, and deletions were common to the two data sets at 4q, 9q, 11q, 13q, 18q, and 19p (GISTIC cutoff q -value <0.25) (Supplemental Table S2). Data set-specific amplifications and deletions were also detected (Supplemental Table S2). Genome-wide, the arm-level copy number alteration frequency correlations were highly significant between the two data sets at both amplified loci (Pearson $r = 0.77$, $P < 0.0001$) and at deleted loci ($r = 0.83$, $P < 0.0001$) (Supplemental Table S2). At loci where intermittent copy number peak deviations were detected in the two data sets, deviation loci were discordant (GISTIC cutoff q -value <0.25) (Supplemental Figures S1 and S2). Although peak regions observed in both data sets do not precisely

Figure 2 Spectra and distribution of ovarian clear cell carcinoma (OCCC)-associated somatic mutations. The frequency of nonsynonymous, truncation, and splicing variants in 3228 OCCC-associated somatic mutations (**A**) and the nucleotide-level mutation spectrum of 3007 single-nucleotide mutations (**B**) are shown. The number of mutated loci for each gene (**C**) and the frequency of mutated genes (percentage of patients with at least one mutation present) in the 39 OCCC samples (**D**) are presented. Genes displayed are those from a prior pan-cancer study.^{23–25} For loci analysis, missense and truncating mutations were plotted in blue and orange bars, respectively. Mutated positions of the most frequently truncated gene *ARID1A* (**E**) and activated gene *PIK3CA* by nonsynonymous substitution (**F**) are drawn with their functional domain structure. The number of missense and truncating mutations in each position are indicated with green and red circles, respectively.

correlate to one another, a variety of genes that are known to act as cancer drivers can be jointly observed to have mutation (GISTIC FDR $q < 0.25$) (Supplemental Figures S1 and S2).

We additionally examined the intra-data set copy number correlation between pairs of chromosome arms that contained genes that are hypothesized to exhibit a coordinated response. Despite correlations that are yielded via calculations, the correlations are spurious and did not yield actionable hypotheses (Supplemental Figures S3 and S4).

Mutation Pathway and SNV-CNV Integrated Analysis

The STRING database³⁴ was used to map the 426 OCCC-associated genes containing multiple functional variants

onto PPI data. Sixty-one genes were found to be involved a stringently defined PPI network (confidence score >0.95) (Figure 4 and Supplemental Table S3) and screened for significant enrichment in pathway-functional groups ($P < 0.05$) (Supplemental Table S4). As a result, two sufficiently sized networks with a total of 46 genes were annotated into four categorical groups that represented cancer biology, namely, cell proliferation, DNA repair and cell cycle checkpointing, chromatin remodeling (Figure 4A), and cytoskeletal organization (Figure 4B).

The network obtained recapitulates key known oncogenes, including, for example, *PIK3CA*, *KRAS*, and *PPP2R1A* involved in cell proliferation. Members of the SWI/SNF complex, including the critical gene *ARID1A*, are

Figure 3 Copy number analysis of ovarian clear cell carcinomas (OCCC). Copy number alterations calculated from 39 exomes (A) and 112 array comparative genomic hybridization samples (<https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE58342) (B) of patients with OCCC. The x axis is the chromosomal position, and the y axis is the mean copy number gain (red) or loss (blue) across each data set. Notable cancer-related genes located in the amplified or deleted loci are indicated by gene symbols with red and blue letters, respectively.

also in the network. Figure 4 gives a comprehensive view of how the pathways that contain highly mutated *PIK3CA* and *ARID1A* are connected by multiple routes and which genes are the intermediates in those routes.

Key driver genes *ARID1A*, *PIK3CA*, *PIK3R1*, and *KRAS*, among 19 total genes (41%), were present in both the IntOGen and pan-cancer study gene list and our derived interaction network (Supplemental Table S5 and Supplemental Figure S5).

Integration of SNV and CNV analyses revealed that identified somatic mutations and CNVs were substantially enriched on the *KRAS-PIK3CA-AKT1-PTEN* pathway, the *MYC-CDK2/4-RB1* pathway, and the aforementioned components of the SWI/SNF complex. These genetic alterations on the two pathways and remodeling complex were observed in 82%, 79%, and 85% of samples, respectively (Figure 5).

Quality Assessment for Somatic Mutation Genes

We checked whether matched tumor-normal pairs cases contained significantly different mutational landscape behavior than tumor-only samples (Supplemental Table S6). The result was negative (Fisher exact test). This finding reiterates our qualification of considering detected mutations to be somatic in nature.

The somatic mutations both in 16 paired cases and 23 unpaired cases contain *ARID1A*, *PIK3CA*, *ARID1B*, *ARID4B*, *KRAS*, *PTEN*, *CREBBP*, and *PIK3R1*, which were

also listed as part of the 19 common genes of our pathway analyses. Although *PPP2R1A* was found only in unpaired cases, it is reported to be a key somatic mutation gene in OCCC¹⁰ (Supplemental Table S7).

Moreover, VAF in truncating and missense mutations was examined for the 426 candidate somatic mutation genes, visualized in Supplemental Figure S6. The VAFs are cross-checked against sequence read counts to warn against bias during handling. Neither inverse nor strong correlation was found in the measurements. The distribution of VAFs appeared to be roughly uniform. One difference that was detectable was the coverage (read counts) in genes with truncation mutations versus those with missense mutations (mean, 81.9 versus 95.0, $P = 0.002$ by t -test).

Correlation between *ARID1A* Mutation and Its Protein Product BAF250a Expression

The correlation between *ARID1A* mutations with or without deletion and the expression of BAF250a, *ARID1A*'s protein product, was confirmed by using immunoreactivity (Supplemental Figure S7) with per-patient analyses shown in Supplemental Table S8. For *ARID1A* mutations with no deletions, a tendency for correlation with loss of expression in BAF250a was observed (Supplemental Figure S7A). After the inclusion of deletions, the association became statistically significant (Supplemental Figure S7B). Interestingly, of our

Figure 4 The connected landscape of somatic mutation in ovarian clear cell carcinoma (OCCC). Genes enriched in somatic mutations were cross-referenced against the STRING protein interaction database, and networks that included more than five genetic members were retained. Nodes are colored by significant functional pathways resulting from Database for Annotation, Visualization and Integrated Discovery analyses. Mutational events occurred at cancer-associated functional pathways. **A:** The network uncovers links between the proliferation and chromatin organization pathways. **B:** A separate, highly connected network of mutated cytoskeletal organization genes is also revealed.

39 cases, only two cases had a biallelic *ARID1A* mutation. They demonstrated complete loss of BAF250a immunoreactivity. Obviously, a much larger patient population with biallelic mutation is required to substantiate this finding.

PTEN Analysis

Aside from mutation, PTEN expression loss is known to occur because of epigenetic silencing.⁴⁰ Therefore, the possible routes of loss of expression are SNV, CNV, and epigenetic silencing, and because large numbers of SNVs and CNVs were not detected in our samples, results suggested epigenetic silencing was a factor. Experiments using

immunohistochemistry revealed an 18% loss of expression of PTEN.

In our study, two cases with *PTEN* missense mutation had positive expression of PTEN IRS. One case with *PTEN* deletion had loss of expression of PTEN. Previous reports of PTEN loss of expression varied from 20% to 51%, depending on the cutoff level used to evaluate the IRS.^{41,42}

Comparison of Copy Number by Exome Sequencing versus SNP Array

For the subset of 12 OCCC samples with matching SNP arrays downloaded from Gene Expression Omnibus

Figure 5 The integrated analysis of somatic mutations and CNVs. Heterozygous and homozygous somatic mutations and copy number amplification and deletion are represented with yellow, green, red, and blue, respectively. These genomic alterations are enriched in KRAS-PIK3CA-AKT1-PTEN and MYC-CDK2/4-RB1 signaling pathways, as well as the ARID1A-ARID1B-SMARCA2-SMARCA4 components of the switch/sucrose nonfermentable (SWI/SNF) complex.

(<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE30311), matched array-exome samples were compared side by side for global similarity. Despite different underlying technologies and resolutions, the measurements are strongly correlated (data not shown).

Discussion

Full exome analysis of OCCC has been almost nonexistent thus far, and as a result of SNV, CNV, and combined analyses on a considerably sized sample, we have charted a first draft of the genome-wide events occurring in this ovarian cancer subtype. In the mutational spectrum of 3007 total base substitution mutations among 39 OCCC tumors, C to T and T to C substitutions were dominant (Figure 2B). It has been reported that such substitutions exhibit strong positive correlations with age in most cancer types.⁴³

Four hundred twenty-six genes were identified that were repeatedly mutated in the OCCC samples. Carcinogenesis drivers *ARID1A* (tumor suppression) and *PIK3CA* (oncogene) somatic mutations were observed in 62% and 54% of the OCCC samples, which is consistent with previous reports in human^{5,7,8} and mice.^{44,45} *ARID1A*, belonging to a family of ATP-dependent chromatin remodelers known as the SWI/SNF complex, has been established as a tumor suppressor gene through the discovery of recurrent inactivating *ARID1A* mutations in a broad spectrum of cancers, including endometriosis without cancer.^{46,47} Inactivating *ARID1A* through somatic mutations and/or other epigenetic mechanisms results in loss of gatekeeper and caretaker functions in cells, thus promoting tumor initiation.⁴⁸

The frequency of *PPP2R1A* somatic mutations (10%) was also concordant with a separate targeted analysis in human endometrial and ovarian carcinomas.¹⁰ Interestingly, somatic mutations of *PIK3CA* and *PIK3R1* genes were mutually exclusive in our data set (Figure 5). To date, such an observation was reported for endometrial cancer⁴⁹ but not for OCCC.

KMT2C/MLL3 and *ARID1B*, related to chromatin modification and chromatin remodeling, respectively, were genes for which we identified somatic mutations in 15% and 10% of OCCC samples, respectively. These associations have not been previously reported.⁵⁰ The mutations were truncations. Evidence of frequent mutations involved in chromatin remodeling and chromatin modification has accumulated in various types of cancer, and chromatin-associated mutations are thought to contribute to tumorigenesis via their effect on gene expression.⁵⁰

The *ARID1A* and *ARID1B* genes have complementary roles to each other and have been reported to be co-mutated.^{51,52} Mutation profiles obtained in this study contained co-mutation. This is in contrast to exclusive mutations detected for *PIK3CA* and *PIK3R1* (Figure 5).

We also detected somatic mutations in *TTN*, *AHNAK2*, and *AHNAK* genes in 23%, 18%, and 10% of samples,

respectively (Supplemental Table S1). These genes have been reported as frequently mutated and possibly driver genes in many cancers,^{25,50} but their functions are unclear. Because the *TTN* gene contains many repetitive sequences in its coding regions, the high frequency of somatic mutations of this gene is difficult to interpret.⁶ From the PPI network analysis, *TTN* has high-confidence interactions with six genes, containing characteristics of a hub gene (Figure 4). DAVID analysis revealed that *TTN* and four of its interaction partners are included in a 6-member actin-binding group (Supplemental Table S5); we consider *TTN* might play a role in tumor development through cell motility.

KRAS, *PTEN*, *AKT1*, *FOXA2*, *STAT3*, and *EP300* were mutated in 5% to 7% of samples. These genes were located together on a *PI3K* mitogenic signaling pathway in the protein-protein network map (Figure 4). Considering that infrequent mutations of multiple genes on the same pathway have been reported to lead to cancer proliferation,^{39,51–53} parallel mutations of these genes could also be important factors to develop OCCC. The network obtained has helped to connect the mitogenic cell proliferation pathway driven by *PIK3CA* with the chromatin remodeling pathway driven by *ARID1A*, expanding the number of possibilities in which infrequent mutations can combine to lead to the phenotype. The resulting network reconfirms our belief that *PIK3CA* is the accelerator for driving cell growth, with the braking mechanism provided by *ARID1A* broken via mutation, combining to be the ideal pathway recipe for OCCC cancerization.

CNV analyses yielded a complementary dimension to the somatic mutation results. The CNV statuses obtained from the exome sequencing data were concordant with the signals from an external validation set of array CGH data (Figure 3). Previously, we reported that two CNVs occurred frequently in OCCC for array data: chr8q amplification and chr13q deletion, where oncogene *MYC* and tumor suppressor gene *RB1* are located, respectively.³⁰ The results herein largely reproduce the previous report,³⁰ indicating that exome sequencing has been substantially effective to detect not only somatic mutations but also CNVs from a single experimental data source. The high reproducibility of copy number aberration found in OCCC samples suggests that CNVs and somatic mutations are critical factors for carcinogenesis and progression of OCCC and may be useful in a joint, complementary fashion to characterize the behavior of an OCCC sample. Compared with a larger array CGH data set, the reproducibility and correlation of arm-level copy number alteration in OCCC exomes were observed (Supplemental Table S3). Although the reproducibility of CNV patterns was observed at arm levels and not at peak loci in the two independent data sets, peak loci nonetheless contained several cancer driver genes (ie, amplified loci contain oncogenes and deleted loci contain tumor suppressor genes). Data match previous reports that accumulation of alteration

in cancer driver genes was observed sporadically (Supplemental Figures S1, S2, S3, and S4).^{13,54}

Our integrated SNV/CNV analysis revealed that almost 80% of alterations occurred in the SWI/SNF complex, KRAS/phosphatidylinositol 3-kinase (PI3K) signaling, and MYC/retinoblastoma (RB) signaling pathways (Figure 5). In recent research involving genes included in these groups, an EZH2 inhibitor has been reported to be effective to treat cancer cells with *ARID1A* (SWI/SNF) mutations.⁵⁵ Similarly, PI3K and mechanistic target of rapamycin (mTOR) inhibitors (KRAS-PI3K) were beneficial for patients with cancer harboring *PIK3CA* alterations.⁵⁶ The CDK2 inhibitor dinaciclib (MYC/RB) has recently begun evaluation for its effectiveness in treating ovarian tumors.⁵⁷ Our results provide us with a possibility to target these pathways as an OCCC therapy, using methods such as chemogenomics^{58–60} to leverage pathway and family similarity to rapidly identify inhibitors in recent or unpursued targets, such as *ARID1B* or *AHNAK*.

The equivalence of paired and unpaired cases is confirmed when considering mutation profiles in genes with low frequencies of mutation. However, when considering genes with high mutation frequencies (*ARID1A*, *PIK3CA*, *ARID1B*, *KRAS*, *PTEN*, and *PIK3R1*), results were similar. Therefore, we thought our analyses with or without normal counterparts to be an overall reliable projection of the mutation landscape for OCCC.

Strong correlation between the loss of BAF250a protein expression and the presence of *ARID1A* mutations has been reported in OCCC.⁷ In this study, we observed loss of BAF250a immunoreactivity in cases with *ARID1A* mutations and deletions, although the sample size was limited (Supplemental Figure S7). Therefore, we can hypothesize that *ARID1A* deletion might induce a loss of immunoreactivity in BAF250a. On the other hand, immunoreactive cases contained *ARID1A* mutations, so some types of *ARID1A* mutations do not seem to affect protein expression. An increased sample size might lead to clarification of association between *ARID1A* mutation and BAF250a expression.

The current study has some limitation because of the unavailability of normal counterparts in some samples, which raises a possibility of inclusion of false germline mutations through the analysis. Nevertheless, we could successfully reproduce the identification of previously known genomic alterations.⁶ Furthermore, we also revealed novel associations of genomic alterations that occur frequently in the OCCC samples. The protein-product interaction network has generated a new perspective about how some functional groups with mutations are linked to each other by intermediaries (Figure 4). Taken together, this report yields additional evidence of the major factors in OCCC and targets for anticancer drug development.

OCCC is much more frequent in Japan compared with other populations and therefore deserves elevated research efforts. Planning is under way to increase the number of

samples by collaborating with multiple centers of medical research throughout Japan. The effort is essential to deepen and expand knowledge about the subtype and to develop a tailored therapy driven by genetic diagnosis.

In conclusion, tumor-blood whole exome analyses reproduced mutations in known cancer genes and produced novel frequently mutated genes for further investigation. Independent SNV and CNV analyses, as well as their combination, provided insightful complementary views of the genome-wide landscape of OCCC. Elements of the KRAS/PI3K signaling pathway, the MYC/RB signaling pathway, and the SWI/SNF complex were enriched, reconfirming their status as potential therapeutic targets for patients with OCCC.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.06.012>.

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