Identification of novel mutations of ovarian cancer-related genes from RNA-sequencing data for Japanese epithelial ovarian cancer patients

Saya Nagasawa^{1),2)}, Kazuhiro Ikeda¹⁾, Kuniko Horie-Inoue¹⁾, Sho Sato³⁾, Satoru Takeda²⁾, Kosei Hasegawa³⁾ and Satoshi Inoue^{1),4)}

¹⁾ Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Hidaka, 350-1241 Saitama, Japan

²⁾ Department of Obstetrics and Gynecology, Juntendo University, Bunkyo-ku, 113-8431 Tokyo, Japan

³⁾ Department of Gynecologic Oncology, Saitama Medical University International Medical Center, Hidaka 350-1298 Saitama, Japan

⁴⁾ Department of Systems Aging Science and Medicine, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku 173-0015 Tokyo, Japan

Abstract. Ovarian cancer has the highest mortality rate among gynecological cancers. Gene mutations are involved in the carcinogenesis, metastasis, and therapeutic response in ovarian cancer. However, the variety and proportion of gene mutation is not fully analyzed in Japanese ovarian cancer patients, especially, in those with recurrent tumors. In the present study, RNA-sequencing was performed for 32 clinical ovarian specimens obtained from 24 Japanese patients (24 primary cancer specimens and 8 recurrent specimens paired with corresponding primary cancer specimens). Mutations in 24 primary specimens were analyzed by comparing the sequence data mapped on RefSeq genes with those in the public online databases BRCA Exchange, COSMIC, ClinVar, and cBioportal. Mutations were observed in *TP53* in 16 specimens (67%), *BRC1* in 9 (38%), *BRC2* in 13 (54%), *ARID1A* in 3 (13%), *PIK3CA* in 2 (8%), *KRAS* in 1 (4%), *PTEN* in 1 (4%), and *CTNNB1* in 1 (4%), excluding synonymous mutations. Among those identified mutations, 13 of 14 mutations in *TP53*, 10 of 11 mutations of *BRC1*, 10 of 23 mutation positions of *BRC2*, none of 7 mutations of *ARID1A*, 1 mutation of *PIK3CA*, and 1 mutation of *CTNNB1* were consistent with those reported in the public online databases; however, the other mutations identified were novel. Comparison between matched-paired specimens of primary and recurrent tumors revealed the changes of mutational status in expressed RNAs. RNA-sequencing-based mutation analysis will be useful to reveal ethnic differences of gene mutations in ovarian cancer and to understand the contribution of gene mutations to recurrence.

Key words: Ovarian cancer, Mutation, RNA-sequencing, Clear cell carcinoma, High-grade serous carcinoma

OVARIAN CANCER has the highest mortality rate in gynecological cancers and is responsible for the death of approximately 4,500 women per year in Japan [1]. Epithelial ovarian cancer (EOC) is pathologically classified according to the histology as high-grade and low-grade serous carcinoma, clear cell carcinoma (CCC), endometrioid carcinoma (EC), and mucinous carcinoma (MC). High-grade serous carcinoma (HGSC) is the most common histotype of ovarian cancer and is usually diagnosed at an advanced stage, exhibiting aggressive behavior [2]. HGSC occurs in 70% of malignant ovarian cancer in

Caucasian [3]. The characteristics of ovarian cancer among Japanese is rather unique compared with those among Caucasian. Although the proportion of HGSC in Japanese (35%) is lower than that in Caucasian, the proportion of CCC is as high as 24% in Japanese (the second highest proportion of the disease) and only 10% in Caucasian [4]. The proportions of EC (17%) and MC (11%) are also higher in Japanese than those in Caucasian. The difference between histotype proportions of Caucasian and Japanese is possibly caused by the difference of genetic background. The precise mechanism, however, still remains to be clarified.

Somatic mutations in oncogenes have been observed in many human cancers [5]. The Cancer Genome Atlas (TCGA) reported mutations in *TP53*, which are predominant among ovarian cancer, occurring in at least 96% of HGSC specimens; approximately 22% of tumors exhibit-

Submitted Jul. 4, 2019; Accepted Oct. 21, 2019 as EJ19-0283

Released online in J-STAGE as advance publication Nov. 19, 2019

Correspondence to: Satoshi Inoue, Department of Systems Aging Science and Medicine, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, 173-0015, Tokyo, Japan.

E-mail: sinoue07@gmail.com

ed mutations in *BRCA1* and *BRCA2* owing to a combination of germline and somatic mutations [6]. Interestingly, exome analysis for Japanese HGSC patients presented *TP53* mutation ratio as only 76% [7]. On the other hand, the prevalence of pathogenic germline *BRCA1/2* variants in Japanese ovarian cancer patients was not reported to show a large difference compared with that in other ethnicities [8, 9]. *BRCA1* and *BRCA2* play key roles in homologous recombination (HR), which is the primary mechanism for repairing double-stranded DNA breaks. Presumably, HGSC originates from the secretory epithelial cells that have lost their DNA repair systems in the fallopian tubes, whereas CCC and EC can originate from endometriosis. The pathogenesis of endometriosis is multifactorial; however, it may be caused by transtubal dissemination into the peritoneal cavity, leading to endometrial tissue implantation and sustained growth. Pearce *et al.* reported that endometriosis was associated with a significant risk of CCC; 20.2% of patients with CCC reported a history of endometriosis [10]. CCC and EC tend to have wild-type *TP53* and rarely harbor *BRCA1/BRCA2* mutations. In CCC, mutations of the AT-rich interactive domain A (*ARID1A*) and/or phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*) are commonly observed in Caucasian [11]. Japanese ovarian CCC patients were reported to have higher mutation ratio of *ARID1A* (67–78%) compared to Caucasian, while similar or lower mutation ratio of *PIK3CA* (11–50%) compared to Caucasian [12, 13]. In EC, aberrations have been reported in the Wnt signaling pathway involving the somatic mutations of catenin $\beta 1$ (*CTNNB1*) and phosphatase and tensin homologue (*PTEN*) [14]. Ovarian endometrioid carcinoma was reported to harbor mutations of *CTNNB1* in 16–53%, *PTEN* in 14–20%, and *ARID1A* in 30–55% of the cases in Europe [15]. In contrast, mutation ratios of *PTEN* and *ARID1A* among Japanese ovarian EC patients are high as 54% and 92%, respectively [13]. Mutation ratio of *CTNNB1* among Japanese EC patients remains to be examined. MC is thought to be malignantly transformed from borderline mucinous tumor, and the most common molecular lesions are observed in the proto-oncogene *KRAS* (a GTPase [*KRAS*] mutation); this has been consistently identified in 50–65% of patients with MC [16, 17]. Among Japanese patients, *KRAS* mutation ratio in MC cases was similar to that among Caucasian whereas that in CCC cases (25.9%) was much higher than Caucasian (7%) [12, 18].

Only a few number of reports is found concerning the genetic status of recurrent ovarian cancer tumors. In a whole exome-sequencing study in paired primary and recurrent samples from 50 HGSC patients with adjuvant platinum-based chemotherapy, *BRCA1/2* mutations were

maintained between primary and recurrent tumors, except for one pair in which a *BRCA1* reversion mutation was identified in the recurrent sample [19]. In a recent whole exome-sequencing study in paired primary and metastatic/recurrent tumors from 57 ovarian cancer patients including mainly HGSC, known cancer driver mutations such as *BRCA1/2*, *TP53*, and *PIK3CA* were also commonly retained during ovarian cancer evolution and/or chemotherapy exposure [20]. They found that primary tumors transmit 54% of mutations to their corresponding recurrent tumors. Their CNV analysis also revealed that 3q26, 8q23-24, and 14q11 were the most significantly amplified regions across all ovarian cancer groups, and *PIK3CA* amplifications from 3q26 and *c-MYC* amplifications from 8q23-24 were found in some recurrent patients. In a mutation study for *BRCA2*-mutated ovarian cancers, recurrent ovarian cancer that develops cisplatin resistance would harbor secondary *BRCA2* mutations that correct the frameshift caused by mutated *BRCA2* alleles, leading to the acquisition of cisplatin resistance [21].

Compared to the DNA-based exome sequencing, RNA-sequencing analysis is capable of providing information regarding the genes that are currently expressed. However, as mentioned above, there are few reports of RNA-sequencing-based mutation analysis. It is also scarcely investigated whether gene mutation rates or mutations positions of Japanese patients are consistent with online databses based on Caucasian. In the present study, we employed high-throughput RNA-sequencing to identify gene mutations in Japanese ovarian cancer patients. RNA-sequencing followed by the mutation analysis of 32 clinical ovarian specimens obtained from 24 patients (24 primary cancer specimens and 8 recurrent specimens paired with corresponding primary cancer specimens) identified novel mutations in *TP53*, *BRCA1*, *BRCA2*, *ARID1A*, *PIK3CA*, *KRAS*, *PTEN*, *CTNNB1*, and other HR-related genes such as *ATM*. RNA-sequencing-based mutation analysis will be useful to determine the contribution of gene alteration in ovarian cancer and to understand ethnic differences in mutations.

Materials and Methods

Clinical specimens

Experiments using patient data and tissue specimens were approved by the Institutional Review Board of Saitama Medical University International Medical Center (#13-165). Tumor specimens were available from 24 patients who underwent surgery for the following primary ovarian cancers: primary HGSC (15 specimens, S1–S15), primary CCC (6 specimens, C1–C6), primary unclassified carcinomas (UCs; 2 specimens, U1 and U2),

primary EC (1 specimen, E1). Recurrent tumors (8 specimens: S11R-S15R, U1R, U2R and E1R) paired with the corresponding primary tumors (S11-S15, U1, U2, and E1) were obtained from surgery or ascites.

RNA-sequencing

RNA was extracted from freshly frozen tissues using NucleoSpin RNA (Takara, Japan). Quality control was performed using a Bioanalyzer (Agilent, USA), and all RIN (RNA Integrity Number) values were >8. RNA library was prepared using the SureSelect Strand Specific RNA Library Prep Kit (Agilent), and 100-bp paired-end RNA-sequencing was performed using the HiSeq2500® (Illumina, USA). rRNA and adapter sequence were deleted from the FASTQ data. RNA-sequencing reads were aligned to the human genome assembly hg19 using TopHat based on the Bowtie algorithm, and the mutation positions were identified based on the RefSeq gene model. Gene duplication and raw variants were filtered using the GATK Best Practice and SNPiR systems (available at https://www.broadinstitute.org/gatk/img/BP_workflow_RNA_3.6.png). SNVs specific to the Japanese population were removed according to the Tohoku University database Integrative Japanese Genome Variation.

Mutation analysis

Mutation data based on RNA-sequencing were compared with those in the online somatic mutation databases BRCA Exchange (available at <https://brcaexchange.org/>), COSMIC (available at <https://cancer.sanger.ac.uk/cosmic>) and cBioportal (available at <https://www.cbioportal.org/>). Mutations were also compared between 8 matched-paired primary and recurrent tumor samples. Synonymous mutations were excluded from these analysis. We performed Combined Annotation-Dependent Depletion (CADD) analysis, which is a prediction tool integrating the information from many various functional gene annotations and condensing this information into a single score [22]. High CADD score indicates a potentially deleterious role of the variant. We did not exclude benign variants and variants of unknown significance.

Results

Mutation analysis for primary ovarian cancer specimens from RNA-sequencing data reveals novel mutations

First, RNA-sequencing was performed for 24 primary ovarian cancer specimens: 15 HGSCs, 6 CCCs, 1 EC, and 2 UCs (Supplementary Table 1). Then, gene mutations observed in *TP53*, *BRCA1*, *BRCA2*, *ARID1A*, *PIK3CA*, *KRAS*, *PTEN*, and *CTNNB1* were compared

with the mutations in these genes reported in the online databases (Fig. 1a) as they were the common mutated genes in ovarian cancer. Mutations were observed in *TP53* in 16 specimens (67%), *BRCA1* in 9 (38%), *BRCA2* in 13 (54%), *ARID1A* in 3 (13%), *PIK3CA* in 2 (8%), *KRAS* in 1 (4%), *PTEN* in 1 (4%), and *CTNNB1* in 1 (4%). The mutation rates in each histotype are shown in Fig. 1b. All data shown in the present study are excluding synonymous mutations.

TP53 mutations were found in 12 of 15 primary HGSCs (80%), 1 of 6 primary CCCs (17%), 1 primary EC (100%), and 2 primary UCs (100%) (Table 1). The mutation positions in *TP53* identified in primary tumors are shown in Fig. 2a and Table 2. Eleven mutation positions were detected in primary tumors, and ten of them were consistent with those reported in the public online database COSMIC or ClinVar: codons 11, 72, 141, 175, 193, 220, 245, 273, and 378 (underlined in Table 2). Notably, R273H which has been known as a hotspot of mutated *TP53* was also detected in the present study. We found a new mutation R267G with a high Combined Annotation Dependent Depletion (CADD) score similar to the level of known mutations such as G245S, R175H, and Y220C, indicating that R267G will also play a potentially deleterious role in tumorigenesis.

BRCA1 mutations were detected in 6 primary HGSCs (40%), 1 primary EC (100%), and 2 primary UCs (100%), but not in CCC (0%) (Table 1). The mutation positions in *BRCA1* are shown in Fig. 2b and Supplementary Table 2. Ten mutation positions were detected in primary tumors, and all of them were found in those reported in the public online database BRCA Exchange or ClinVar: codons 52, 63, 797, 871, 1038, 1183, 1609, 1613, 1628, and 1853 (underlined in Supplementary Table 2).

BRCA2 mutations were found in 9 primary HGSCs (60%), 1 primary CCC (17%), 1 primary EC (100%), and 2 primary UCs (100%) (Table 1). The mutation positions in *BRCA2* are shown in Fig. 2c and Supplementary Table 3. Fifteen mutation positions were detected in primary tumors, and eight of them were consistent with those reported in the public online database BRCA Exchange: codons 372, 819, 991, 1024, 2447, 2466, 2475, and 2729 (underlined in Supplementary Table 3). Among the new mutations detected, E911K has the highest CADD score.

ARID1A mutations were found in 3 primary CCCs (50%) but not in primary HGSCs, EC, and UCs (Table 1). The mutation positions in *ARID1A* are shown in Fig. 3a and Table 3. All five mutation positions detected in primary tumors were not identical to those reported in the public online databases. Among these new mutations, E2098X and R1276X with premature stop codon showed

Fig. 1 Gene mutation analysis of primary ovarian cancer specimens by RNA-sequencing

(a) Schematic representation of mutation analysis in primary ovarian cancer specimens using RNA-sequencing. (b) Gene mutation rates observed in primary ovarian cancer specimens are shown in each histotype.

relatively high CADD score.

PIK3CA mutations were found in 2 primary CCCs (33%), *KRAS* mutation was found in 1 primary HGSC (7%), *PTEN* mutation was found in 1 primary HGSC (7%), and *CTNNB1* mutations were found in 1 primary CCC (17%) (Table 1). These mutation positions are shown in Fig. 3b-e and supplementary Tables 4–7. One mutation position of *PIK3CA* is consistent with a hotspot (H1047R) and one mutation position of *CTNNB1* is registered in ClinVar; however, mutation positions of *KRAS* and *PTEN* were not found in those reported in the public online databases.

Matched-paired specimens reveal novel hotspots of Japanese patients

Next, we performed RNA-seq using 8 recurrent ovarian cancer specimens (Sample No. S11R–S15R, E1R, E2R, and U1R), which are paired to the primary cancers (Sample No. S11–S15, E1, E2, and U1), and searched mutations for these genes (Supplementary Fig. 1a). The mutation rates in each histotype of recurrent tumors are shown in Supplementary Fig. 1b.

In terms of *TP53* mutation, a primary mutation position P72R remained in recurrent pairs of E1, U1, and U2, whereas not in those of HGSCs (Sample No. S11–S15) (Table 2). In addition, S12R, S14R, and U1R received new mutations on *TP53*.

BRCA1 mutations were found in 1 HGSC (Sample No. S15R), 1 EC, and 2 UCs (Sample No. U1R and U2R) (Table 1). Notably, the several primary mutation positions were retained in the recurrent tumors: P871L in E1; E1038G in E1 and U2; K1183R in E1, U1, and U2; and S1613G in E1 and U1 (Supplementary Table 2). The other primary mutation positions of *BRCA1* were not detected in the recurrent specimens. Besides, S15R, U1R, and U2R received new mutations.

BRCA2 mutations were found in 3 HGSCs, 1 EC, and 2 UCs (Table 1). Primary mutation position V2466A remained in the recurrent pairs of E1, U1, and U2, whereas not in those of HGSCs (S11–S15) (Supplementary Table 3). The other primary mutation positions were not detected in the recurrent pairs. Additionally, S11R, S12R, S15R, U1R, and U2R received new mutation positions (Supplementary Table 3).

Table 1 Number of mutations observed in each sample

Status	Case	Pathology	Gene							
			<i>TP53</i>	<i>BRCA1</i>	<i>BRCA2</i>	<i>ARID1A</i>	<i>PIK3CA</i>	<i>KRAS</i>	<i>PTEN</i>	<i>CTNNB1</i>
Primary	S1	Serous	1	0	0	0	0	0	0	0
	S2	Serous	1	0	0	0	0	0	0	0
	S3	Serous	1	0	0	0	0	0	0	0
	S4	Serous	0	1	3	0	0	0	0	0
	S5	Serous	1	1	0	0	0	0	0	0
	S6	Serous	1	0	1	0	0	0	0	0
	S7	Serous	0	1	1	0	0	0	0	0
	S8	Serous	1	0	1	0	0	0	0	0
	S9	Serous	0	0	0	0	0	0	0	0
	S10	Serous	1	0	0	0	0	0	0	0
	S11	Serous	1	0	1	0	0	0	0	0
	S12	Serous	1	0	3	0	0	0	1	0
	S13	Serous	1	1	2	0	0	1	0	0
	S14	Serous	3	2	2	0	0	0	0	0
	S15	Serous	1	1	4	0	0	0	0	0
	C1	Clear	0	0	0	2	1	0	0	0
	C2	Clear	0	0	0	0	0	0	0	0
	C3	Clear	1	0	1	0	1	0	0	0
	C4	Clear	0	0	0	0	0	0	0	0
	C5	Clear	0	0	0	2	0	0	0	0
	C6	Clear	0	0	0	1	0	0	0	1
	E1	Endometrioid	1	4	2	0	0	0	0	0
	U1	Unclassified	1	5	1	0	0	0	0	0
	U2	Unclassified	1	2	1	0	0	0	0	0
Recurrent	S11R*	Serous	0	0	1	0	0	0	0	0
	S12R*	Serous	1	0	3	0	0	0	0	0
	S13R*	Serous	0	0	0	0	0	0	0	0
	S14R*	Serous	1	0	0	0	0	0	0	0
	S15R*	Serous	0	1	1	0	0	0	0	0
	E1R*	Endometrioid	1	4	1	0	0	0	0	0
	U1R*	Unclassified	2	3	2	2	0	0	0	0
	U2R*	Unclassified	1	4	3	0	0	0	0	0

* R means recurrence.

The U1R has two new mutation positions in *ARID1A* (Table 3). In terms of *PIK3CA*, *KRAS*, *PTEN*, and *CTNNB1*, mutations were observed only in primary tumors.

All matched-paired specimens of HGSCs exhibit HR deficiency

We also searched for mutations in other 15 HR-related genes using 5 matched-paired specimens of primary and recurrent HGSCs (Supplementary Table 8). The mutation positions in HR-related genes are shown in Supplementary Table 9. *ATM* mutations were found in 5 primary

Fig. 2 Mutations detected in *TP53*, *BRCA1*, and *BRCA2*

Mutation positions of *TP53* (a), *BRCA1* (b), and *BRCA2* (c) detected in primary ovarian cancer specimens are shown on the domain structures of the genes. Triangles represent mutations identified in the study where red-colored ones are newly identified mutations.

and 4 recurrent HGSCs. Among these mutations, N1983S and M1321I remained in recurrent tumors. *ATR* mutations were detected in 3 primary and 4 recurrent HGSCs where R2425Q and M211T mutations were found in both primary and recurrent tumors. *BLM* mutations were detected in 1 primary and 2 recurrent HGSCs; however, no common mutation position was found. *BRIP1* mutations were found in 4 primary and 3 recurrent HGSCs. Especially, *BRIP1* S919P mutation remained in recurrent pairs of S11, S13 and S14. *CHEK1* I471V nonsynonymous mutations were found in all HGSCs. *FANCA* mutations were found in all HGSCs where G809D and G501S were retained in recurrent tumors. *NBN* E185Q mutations were found in four pairs of primary and recurrent HGSCs. *PALB2* mutations were found in 1 primary and 2 recurrent HGSCs. Three kinds of *WRN* mutations (A136T, L1074F, and C1367R) were found in 5 primary and 4 recurrent HGSCs. Interestingly, less than half of them was identical with those reported in the public online databases although some of them had high CADD scores (Y741X of *ATM*, L783P of *BLM* and C42X of *BRIP1*). Among the analyzed genes, no mutation was found in *CHEK2*, *FANCD2*, *FANCE*, *FANCF*, *RAD51B*, and *RAD51D*.

Discussion

We performed mutation analysis of 24 primary ovarian cancer specimens based on RNA-sequencing and identified novel mutations in *TP53*, *BRCA1*, *BRCA2*, *ARID1A*, *PIK3CA*, *KRAS*, *PTEN*, and *CTNNB1*. Furthermore, comparison between primary and recurrent paired specimens revealed additional gene mutation in *TP53*, *BRCA1*, *BRCA2*, and HR-related genes. In the present study, the expression levels of above-mentioned genes were confirmed by RPKM values, which were not correlated to the detection of mutations or the numbers of mutation positions.

The tumor suppressor gene *TP53* encodes the p53 protein, which plays a role in DNA repair, cell cycle arrest, apoptosis, and other pathways that prevent the development of cancers. More than 90% of HGSCs have *TP53* mutation and half of them are considered to originate from the fimbriae of the fallopian tubes [23]. In the present study, 80% of primary HGSCs had *TP53* mutation, whereas only 40% of recurrent HGSCs had. In comparison of paired HGSC specimens, all primary *TP53* mutations were not detected in their recurrent specimens. This suggests that the HGSC specimens have heterogeneity within the same patient. In addition, cancer

Table 2 Mutation positions of *TP53*

Status	Genome position	Mutation	Genome change	Amino acid change	Exonic Function	Pathology	Subject	CADD score
Primary	chr17: 7579882	C > G	G31C	<u>E11Q*</u>	nonsynonymous SNV	Serous	S8	13.28
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Serous	S11	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Serous	S12	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Serous	S13	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Serous	S14	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Endometrioid	E1	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Unclassified	U1	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Unclassified	U2	8.316
	chr17: 7579328	CT > C	369delA	<u>K120fs*</u>	frameshift deletion	Serous	S14	—***
	chr17: 7578508	C > T	G422A	<u>C141Y*</u>	nonsynonymous SNV	Serous	S10	15.06
	chr17: 7578406	C > T	G524A	<u>R175H*</u>	nonsynonymous SNV	Serous	S3	31
	chr17: 7578406	C > T	G524A	<u>R175H*</u>	nonsynonymous SNV	Serous	S5	31
	chr17: 7578271	T > C	A578G	<u>H193R*</u>	nonsynonymous SNV	Serous	S6	14.03
	chr17: 7578190	T > C	A659G	<u>Y220C*</u>	nonsynonymous SNV	Clear	C3	22.1
	chr17: 7577548	C > T	G733A	<u>G245S*</u>	nonsynonymous SNV	Serous	S2	33
	chr17: 7577139	G > C	C799G	R267G	nonsynonymous SNV	Serous	S15	20.5
	chr17: 7577120	C > T	G818A	<u>R273H*</u>	nonsynonymous SNV	Serous	S1	18.03
	chr17: 7572977	A > G	T1132C	<u>S378P*</u>	nonsynonymous SNV	Serous	S14	14.01
Recurrent	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Endometrioid	E1R**	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Unclassified	U1R**	8.316
	chr17: 7579472	G > C	C215G	<u>P72R*</u>	nonsynonymous SNV	Unclassified	U2R**	8.316
	chr17: 7578490	AC > A	439delG	V147fs	frameshift deletion	Unclassified	U1R**	—***
	chr17: 7577521	TG > T	759delC	T253fs	frameshift deletion	Serous	S12R**	—***
	chr17: 7574003	G > A	C1024T	<u>R342X*</u>	stopgain	Serous	S14R**	37

* Underline means registered in COSMIC or ClinVar.

** R means recurrence.

*** — means there is no CADD score.

cells containing such mutations may be eradicated by therapy. In contrast, UC and EC retained their primary *TP53* mutation (P72R) in the recurrent specimens, suggesting a hotspot mutation of Japanese UC and EC patients.

Generally, *BRCA* germline mutations are observed in 13%–16% and somatic mutations are detected in approximately 6% of HGSCs [24]. The hallmarks of *BRCA1/BRCA2*-associated ovarian carcinomas include sensitivity to platinum chemotherapy, improvement of overall survival [25–30], and sensitivity to poly(ADP-ribose) polymerase inhibitors (PARP-i) [31, 32]. In addition to *BRCA1/BRCA2* mutations, other HR-related gene mutations may increase sensitivity to DNA-damaging agents. *In vitro* studies demonstrate that deficiency in other HR proteins such as ATM, CHEK1, CHEK2, NBN, and

RAD51D also confer sensitivity to PARP-i [33, 34]. PARP-i are active agents in ovarian carcinomas with germline *BRCA1/BRCA2* mutations but also in a subset of “sporadic” recurrent platinum-sensitive ovarian carcinomas [32]. Indeed, TCGA reported HR defects in approximately 50% of HGSCs [6]. In the present study, 40% of primary HGSCs and 20% of recurrent HGSCs possessed *BRCA1* mutation, and 60% of primary HGSCs and 60% of recurrent HGSCs showed *BRCA2* mutation. Notably, the incident of *BRCA* mutation was much higher among Japanese women than those reported from Western countries. Sugano *et al.* reported the possibility of higher incident of *BRCA* mutation among Japanese patients compared to non-Ashkenazi individuals in the US [35]. All of the primary *BRCA1* and *BRCA2* mutations of HGSCs were not detected in the recurrent speci-

Fig. 3 Mutations detected in *ARID1A*, *PIK3CA*, *KRAS*, *PTEN*, and *CTNNB1*

Mutation positions of *ARID1A* (a), *PIK3CA* (b), *KRAS* (c), *PTEN* (d), and *CTNNB1* (e) detected in primary ovarian cancer specimens are shown on the domain structures of the genes. Triangles represent mutations identified in the study where red-colored ones are newly identified mutations.

Table 3 Mutation positions of *ARID1A*

Status	Genome position	Mutation	Genome change	Amino acid change	ExonicFunction	Pathology	Subject	CADD score
Primary	chr1: 27094372	AT > A	3081delT	Y1027fs	frameshift deletion	Clear	C1	—**
	chr1: 27099947	C > T	C3826T	R1276X	stop gain	Clear	C5	41
	chr1: 27106649	G > T	G6260T	G2087V	nonsynonymous SNV	Clear	C6	17.09
	chr1: 27106670	GC > G	6282delC	C2094fs	frameshift deletion	Clear	C5	—**
	chr1: 27106681	G > T	G6292T	E2098X	stop gain	Clear	C1	43
Recurrent	chr1: 27023063	GA > G	170delA	E57fs	frameshift deletion	Unclassified	U1R*	—**
	chr1: 27023085	G > A	G191A	G64E	nonsynonymous SNV	Unclassified	U1R*	17.07

* R means recurrence.

** — means there is no CADD score.

mens. In contrast, all UCs and ECs retained one primary *BRCA1* mutation (K1183R) and one primary *BRCA2* mutation (V2466A). These results suggest that primary HGSC with *BRCA1* or *BRCA2* mutation is sufficiently controlled by the standard first-line therapy and that tumors without *BRCA* mutation exhibit recurrence. However, primary HGSC specimens retained their HR-related gene mutations after recurrence: *ATM*, *ATR*, *BRIP1*, *CHEK1*, *FANCA*, *NBN*, *PALB2*, and *WRN*. Therefore, in

recurrent HGSCs not retaining *BRCA1* or *BRCA2* mutations, treatment using PARP-i may be effective due to their HR-defect status. Additionally, mutations of L63X in *BRCA1* among Japanese ovarian cancer patients, and mutations of M1628T in *BRCA1* and K2729N in *BRCA2* among Korean ovarian cancer patients were previously reported [35, 36]. The findings of this study suggest that L63X, M1628T, K1183R, and L771L may be new hot-spots of *BRCA1* mutations, and L1521L, V2466A, and

K2729N may be new hotspots of *BRCA2* mutations in East Asian patients.

Regarding CCCs, the main driver mutations are assumed to be the loss-of-function of *ARID1A* or the activation of the phosphoinositol 3-kinase (PI3K) pathway [37–40]. We observed the proportion of *ARID1A* in 50% CCCs and *PIK3CA* mutations in 33% CCCs. Two patients exhibited *ARID1A* mutation, one patient exhibited *PIK3CA* mutation, and the other one patient exhibited both of these mutations. Wiegand *et al.* found that 46% of patients with CCC had somatic truncating or missense mutations of *ARID1A* [37]. *ARID1A* encodes the protein BAF250a, which acts as an accessory subunit of the SWItch/Sucrose Non-Fermentable chromatin remodeling complex, which regulates several cellular processes, including tumor suppression. *ARID1A* mutations result in the functional absence or decreased expression of BAF250a. Endometriotic lesions that are adjacent to endometrioid lesions also exhibit a loss-of-function BAF250a mutation, suggesting that this mutation may be an early step in the transformation [38]. *PIK3CA* encodes for p110a, which forms the catalytic subunit of the phosphorylation molecule PI3K [39]. *PIK3CA* mutations were found to be present in 40% of patients with CCCs and comprised missense and cluster mutations in exons 9 and 20. The activation of the PI3K/AKT pathway regulates downstream target genes that inhibit apoptosis and promote cell proliferation and also opposes the action of *PTEN*, a tumor suppressor that acts as a negative regulator for the aforementioned pathway [40].

Atypical endometriosis is a direct precursor of EC, with 40% of carcinomas presenting concurrent endometriosis [41]. EC is likely to be found at an early stage and has been reported to have somatic mutations of *CTNNB1*, *PTEN*, and *PIK3CA* [42]. *CTNNB1* is a gene that encodes β -catenin, a protein that plays an important role in cell adhesion and cell proliferation [43]. *CTNNB1* mutations lead to squamous cell differentiation, which is a histopathologic feature of endometrioid cancers [42]. However, in this study, *CTNNB1* mutations were detected only in one primary CCC but not in EC. *KRAS* and *PTEN* mutations were detected in one primary HGSC but not in EC. In the present study, the mutation rates and their common histotypes of *KRAS*, *PTEN*, and *CTNNB1* were different from the existing data mainly based on Western countries.

The majority of mutations observed in HR-related genes were not detected in the public online database COSMIC. Notably, in this study, we newly identified 3 mutation positions in *ATM*, 3 in *ATR*, 2 in *BLM*, 2 in *BRIP1*, 3 in *FANCA*, 1 in *PALB2*, and 2 in *WRN*. We identified the possible new hotspots of *ATM* (N1983S),

ATR (M211T and R2425Q), *BRIP1* (S919P), *CHEK1* (I471V), *FANCA* (T266A, G501S, and G809D), *NBN* (E185Q), and *WRN* (L1074F).

Unlike DNA sequencing, RNA-sequencing provides the information for transcripts after RNA processing and enables to analyze simultaneously both gene mutation and expression. The data generated by RNA-sequencing have advantages to more easily identify allele-specific expression, alternatively spliced genes, and non-coding RNAs. Therefore, RNA-sequencing is capable of uncovering novel mutations in transcriptionally active genes. Sun *et al.* reported that RNA-sequencing based on commonly used analysis programs with variant and somatic mutation callers can be reliably used for both single nucleotide variation (SNV) and indel mutation detection that can be used for clinical management [44]. Nevertheless, there are limitations of RNA-sequencing for analyzing transcriptionally unexpressed genes and posttranscriptionally silenced genes exemplified by nonsense-mediated mRNA decay. Based on a comparison study of exome-sequencing and RNA-sequencing for lung cancer samples by O'Brien *et al.*, they discussed the differences of the two methods and discrepancies in SNV calling [45]. While SNVs detected by exome-sequencing are derived from captured exon regions, RNA-sequencing could also detect SNVs outside the boundary of the exome-seq capture regions. SNVs detected by RNA-sequencing also show mutational signature of RNA editing especially in 3'-untranslated region, as exemplified by the deamination of adenosine that converts to inosine, resulting in adenosine to guanine conversion. In the present study, we consider that RNA editing would have minimal contribution to the development of novel variants, as we basically focus on coding regions of cancer-related genes. The results by O'Brien *et al.* indicate that RNA-sequencing could be also a practical technology platform to detect SNVs together with transcript expression, while we need to remind that some SNVs detected by RNA-sequencing would be unique and not observed in exome-seq [45].

The present mutation study based on RNA-sequencing showed that 13 of 14 mutations in *TP53*, 10 of 11 mutations of *BRCA1*, 10 of 23 mutation positions of *BRCA2*, none of 7 mutations of *ARID1A*, 1 mutation of *PIK3CA*, and 1 mutation of *CTNNB1* were consistent with those reported in the public online databases, which were basically constructed by exome-sequencing. This suggests the reliability of mutation analysis using RNA-sequencing data. Notably, a number of novel mutations were identified as putative specific gene alterations in Japanese patients. We examined CADD score for each mutation and found that some of the novel mutations we identified have high CADD score, suggesting their

pathological roles in ovarian cancer. Nevertheless, the precise clinical relevance for each mutation remains to be clarified, thus we may not exclude potential benign variants and variants of unknown significance from the gene mutation list in the present study. Moreover, future gain/loss-of-function study of genes with differential expression will help in further elucidating histotype-specific molecular mechanisms. RNA-sequencing-based mutation analysis is useful to determine the contribution of gene mutation in ovarian cancer and to understand ethnic differences in mutations.

Acknowledgements

We thank Mr. W Sato, Mr. T Oishi, and Ms. A Iwasa for their technical assistance. This work was partially

supported by Grant of the Support Project of Strategic Research Center in Private Universities from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; by the Practical Research for Innovative Cancer Control (JP18ck0106194) and the Project for Cancer Research And Therapeutic Evolution (P-CREATE, JP18cm0106144) from Japan Agency for Medical Research and development, AMED; by Grants from the Japan Society for the Promotion of Science, Japan (16K09809, 16K15496, 17H04205); by the Takeda Science Foundation.

Disclosure

The authors declare that they have no competing interests.

References

- Center for Cancer Control and Information Services, National Cancer Center, Japan. Projected Cancer Statistics, 2018. (https://ganjoho.jp/en/public/statistics/short_pred.html)
- Gurung A, Hung T, Morin J, Gilks CB (2013) Molecular abnormalities in ovarian carcinoma: clinical, morphological and therapeutic correlates. *Histopathology* 62: 59–70.
- Reid BM, Permuth JB, Sellers TA (2017) Epidemiology of ovarian cancer: a review. *Cancer Biol Med* 14: 9–32.
- Yamagami W, Nagase S, Takahashi F, Ino K, Hachisuga T, *et al.* (2017) Clinical statistics of gynecologic cancers in Japan. *J Gynecol Oncol* 28: e32.
- Weinstein IB (2002) Cancer. Addiction to oncogenes—the Achilles heel of cancer. *Science* 297: 63–64.
- Cancer Genome Atlas Research Network (2011) Integrated genomic analyses of ovarian carcinoma. *Nature* 474: 609–615.
- Hayano T, Yokota Y, Hosomichi K, Nakaoka H, Yoshihara K, *et al.* (2014) Molecular characterization of an intact p53 pathway subtype in high-grade serous ovarian cancer. *PLoS One* 9: e114491.
- Hirasawa A, Imoto I, Naruto T, Akahane T, Yamagami W, *et al.* (2017) Prevalence of pathogenic germline variants detected by multigene sequencing in unselected Japanese patients with ovarian cancer. *Oncotarget* 8: 112258–112267.
- Enomoto T, Aoki D, Hattori K, Jinushi M, Kigawa J, *et al.* (2019) The first Japanese nationwide multicenter study of BRCA mutation testing in ovarian cancer: CHARACTERizing the cross-sectional approach to Ovarian cancer geneTic TESting of BRCA (CHARLOTTE). *Int J Gynecol Cancer* 29: 1043–1049.
- Pearce CL, Templeman C, Rossing MA, Lee A, Near AM, *et al.* (2012) Association between endometriosis and risk of histological subtypes of ovarian cancer: a pooled analysis of case-control studies. *Lancet Oncol* 13: 385–394.
- Mabuchi S, Sugiyama T, Kimura T (2016) Clear cell carcinoma of the ovary: molecular insights and future therapeutic perspectives. *J Gynecol Oncol* 27: e31.
- Shibuya Y, Tokunaga H, Saito S, Shimokawa K, Katsuoka F, *et al.* (2018) Identification of somatic genetic alterations in ovarian clear cell carcinoma with next generation sequencing. *Genes Chromosomes Cancer* 57: 51–60.
- Ishikawa M, Nakayama K, Nakamura K, Ono R, Sanuki K, *et al.* (2018) Affinity-purified DNA-based mutation profiles of endometriosis-related ovarian neoplasms in Japanese patients. *Oncotarget* 9: 14754–14763.
- Cho KR, Shih IeM (2009) Ovarian cancer. *Annu Rev Pathol* 4: 287–313.
- Gadducci A, Lanfredini N, Tana R (2014) Novel insights on the malignant transformation of endometriosis into ovarian carcinoma. *Gynecol Endocrinol* 30: 612–617.
- Gemignani ML, Schlaerth AC, Bogomolny F, Barakat RR, Lin O, *et al.* (2003) Role of KRAS and BRAF gene mutations in mucinous ovarian carcinoma. *Gynecol Oncol* 90: 378–381.
- Mackenzie R, Kommoss S, Winterhoff BJ, Kipp BR, Garcia JJ, *et al.* (2015) Targeted deep sequencing of mucinous ovarian tumors reveals multiple overlapping RAS-pathway activating mutations in borderline and cancerous neoplasms. *BMC Cancer* 15: 415.
- Takenaka M, Saito M, Iwakawa R, Yanaihara N, Saito M, *et al.* (2015) Profiling of actionable gene alterations in ovarian cancer by targeted deep sequencing. *Int J Oncol* 46: 2389–2398.
- Patel JN, Braicu I, Timms KM, Solimeno C, Tshiaba P, *et al.* (2018) Characterisation of homologous recombination deficiency in paired primary and recurrent high-grade serous ovarian cancer. *Br J Cancer* 119: 1060–1066.
- Li C, Bonazzoli E, Bellone S, Choi J, Dong W, *et al.* (2019) Mutational landscape of primary, metastatic, and

- recurrent ovarian cancer reveals c-MYC gains as potential target for BET inhibitors. *Proc Natl Acad Sci U S A* 116: 619–624.
21. Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, *et al.* (2008) Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* 451: 1116–1120.
 22. Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, *et al.* (2014) A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 46: 310–315.
 23. Gurung A, Hung T, Morin J, Gilks CB (2013) Molecular abnormalities in ovarian carcinoma: clinical, morphological and therapeutic correlates. *Histopathology* 62: 59–70.
 24. Shaw PA, McLaughlin JR, Zweemer RP, Narod SA, Risch H, *et al.* (2002) Histopathologic features of genetically determined ovarian cancer. *Int J Gynecol Pathol* 21: 407–411.
 25. Alsop K, Fereday S, Meldrum C, deFazio A, Emmanuel C, *et al.* (2012) BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. *J Clin Oncol* 30: 2654–2663.
 26. Bolton KL, Chenevix-Trench G, Goh C, Sadetzki S, Ramus SJ, *et al.* (2012) Association between BRCA1 and BRCA2 mutations and survival in women with invasive epithelial ovarian cancer. *JAMA* 307: 382–390.
 27. Chetrit A, Hirsh-Yechezkel G, Ben-David Y, Lubin F, Friedman E, *et al.* (2008) Effect of BRCA1/2 mutations on long-term survival of patients with invasive ovarian cancer: the national Israeli study of ovarian cancer. *J Clin Oncol* 26: 20–25.
 28. Tan DS, Rothermundt C, Thomas K, Bancroft E, Eeles R, *et al.* (2008) “BRCAness” syndrome in ovarian cancer: a case-control study describing the clinical features and outcome of patients with epithelial ovarian cancer associated with BRCA1 and BRCA2 mutations. *J Clin Oncol* 26: 5530–5536.
 29. Vencken PM, Kriege M, Hoogwerf D, Beugelink S, van der Burg ME, *et al.* (2011) Chemosensitivity and outcome of BRCA1- and BRCA2-associated ovarian cancer patients after first-line chemotherapy compared with sporadic ovarian cancer patients. *Ann Oncol* 22: 1346–1352.
 30. Yang D, Khan S, Sun Y, Hess K, Shmulevich I, *et al.* (2011) Association of BRCA1 and BRCA2 mutations with survival, chemotherapy sensitivity, and gene mutator phenotype in patients with ovarian cancer. *JAMA* 306: 1557–1565.
 31. Audeh MW, Carmichael J, Penson RT, Friedlander M, Powell B, *et al.* (2010) Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial. *Lancet* 376: 245–251.
 32. Gelmon KA, Tischkowitz M, Mackay H, Swenerton K, Robidoux A, *et al.* (2011) Olaparib in patients with recurrent high-grade serous or poorly differentiated ovarian carcinoma or triplenegative breast cancer: a phase 2, multicentre, open-label, non-randomised study. *Lancet Oncol* 12: 852–861.
 33. Loveday C, Turnbull C, Ramsay E, Hughes D, Ruark E, *et al.* (2011) Germline mutations in RAD51D confer susceptibility to ovarian cancer. *Nat Genet* 43: 879–882.
 34. McCabe N, Turner NC, Lord CJ, Kluzek K, Bialkowska A, *et al.* (2006) Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res* 66: 8109–8115.
 35. Sugano K, Nakamura S, Ando J, Takayama S, Kamata H, *et al.* (2008) Cross-sectional analysis of germline BRCA1 and BRCA2 mutations in Japanese patients suspected to have hereditary breast/ovarian cancer. *Cancer Sci* 99: 1967–1976.
 36. Choi MC, Jang JH, Jung SG, Park H, Joo WD, *et al.* (2018) Unclassified variants of BRCA1 and BRCA2 in Korean patients with ovarian cancer. *Int J Gynecol Cancer* 28: 308–315.
 37. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, *et al.* (2010) ARID1A mutations in endometriosis associated ovarian carcinomas. *N Engl J Med* 363: 1532–1543.
 38. Worley MJ Jr, Liu S, Hua Y, Kwok JS, Samuel A, *et al.* (2015) Molecular changes in endometriosis-associated ovarian clear cell carcinoma. *Eur J Cancer* 51: 1831–1842.
 39. Kuo KT, Mao TL, Jones S, Veras E, Ayhan A, *et al.* (2009) Frequent activating mutations of PIK3CA in ovarian clear cell carcinoma. *Am J Pathol* 174: 1597–1601.
 40. Rahman M, Nakayama K, Rahman MT, Nakayama N, Ishikawa M, *et al.* (2012) Clinicopathologic and biological analysis of PIK3CA mutation in ovarian clear cell carcinoma. *Hum Pathol* 43: 2197–2206.
 41. Terada T (2012) Endometrioid adenocarcinoma of the ovary arising in atypical endometriosis. *Int J Clin Exp Pathol* 5: 924–927.
 42. Kurman RJ, Shih IeM (2011) Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer—Shifting the paradigm. *Hum Pathol* 42: 918–931.
 43. Guan L, Zhu S, Han Y, Yang C, Liu Y, *et al.* (2018) Knockout of CTNNB1 by CRISPR-Cas9 technology inhibits cell proliferation through the Wnt/ β -catenin signaling pathway. *Biotechnol Lett* 40: 501–508.
 44. Sun Z, Bhagwate A, Prodduturi N, Yang P, Kocher JA (2017) Indel detection from RNA-seq data: tool evaluation and strategies for accurate detection of actionable mutations. *Brief Bioinform* 18: 973–983.
 45. O’Brien TD, Jia P, Xia J, Saxena U, Jin H, *et al.* (2015) Inconsistency and features of single nucleotide variants detected in whole exome sequencing *versus* transcriptome sequencing: a case study in lung cancer. *Methods* 83: 118–127.