Germline and somatic mutations of homologous recombination-associated genes in Japanese ovarian cancer patients

Kentaro Sugino¹,⁴, Ryo Tamura¹,⁴, Hirofumi Nakaoka²,⁴, Nozomi Yachida¹, Manako Yamaguchi², Yutaro Mori¹, Kaoru Yamawaki², Kazuaki Suda¹, Tatsuya Ishiguro¹, Sosuke Adachi², Masanori Isobe¹, Masayuki Yamaguchi², Katsunori Kashima¹, Teiichi Motoyama³, Ituro Inoue², Kosuke Yoshihara⁶,²,¹* & Takayuki Enomoto¹

We explored the frequency of germline and somatic mutations in homologous recombination (HR)-associated genes in major histological types of ovarian cancer. We performed targeted sequencing to assess germline and somatic mutations of 16 HR-associated genes and 4 mismatch repair (MMR) genes among 207 ovarian cancer patients (50 high-grade serous carcinomas (HGSC), 99 clear cell carcinomas (CCC), 39 endometrioid carcinomas (EC), 13 mucinous carcinomas (MC), and 6 low-grade serous carcinomas (LGSC)). Germline or somatic mutations of HR-associated genes were detected in 44% of HGSC, 28% of CCC, 23% of EC, 16% of MC, and 17% of LGSC patients. The profile of HR-associated gene mutations was remarkably different among each histological type. Germline BRCA1/2 mutations were frequently detected in HGSC and were rarely observed in CCC, EC, and MC patients. ATM somatic mutation was more frequently detected in CCC (9%) and EC patients (18%) than in HGSC patients (4%). There was a positive correlation between MMR gene mutations and HR-associated gene mutations (p = 0.0072). Our findings might be useful in selection of ovarian cancer patients that should be treated with PARP inhibitors.

Recently, the prevalence of homologous recombination (HR)-associated gene mutations among many tumor types has been characterized¹⁻². In particular, HR pathway alterations are most frequently observed in high-grade serous ovarian carcinoma (HGSC) and breast cancer³. It is well-known that around half of HGSC patients exhibit HR deficiency⁴⁻⁵. HR deficiency is also associated with response to platinum-based chemotherapies in patients with ovarian cancer⁶, and germline BRCA1 and BRCA2 mutations, which are representative alterations causing HR deficiency, are undoubtedly associated with improved prognosis in advanced-stage ovarian cancers⁷. In a retrospective analysis, Pennington et al.⁸ have found that the ovarian cancer patients with germline or somatic mutations in 13 HR-associated genes (BRCA1, BRCA2, ATM, BARD1, BRIP1, CHEK1, CHEK2, FAM175A, MRE11A, NBN, PALB2, RAD51C, and RAD51D) had higher platinum sensitivity and prolonged overall survival than those without HR-associated gene mutations.

Clinical use of PARP inhibitors that induces synthetic lethality in HR deficient (HRD) cancer cells has a great impact on treatment strategies for ovarian cancer⁹. Niraparib maintenance therapy has shown prolonged progression-free survival (PFS) in platinum-sensitive, recurrent ovarian cancer patients with HRD⁹. Rucaparib maintenance has also improved PFS in platinum-sensitive, recurrent ovarian cancer patients with HRD¹⁰. However, the majority of subjects in clinical trials of PARP inhibitors were type II ovarian cancer patients⁹⁻¹³, and thus, the efficacy of PARP inhibitors for type I ovarian cancer, such as clear cell or low-grade endometrioid types, remains unclear. To date, the frequency of germline and somatic HR-associated gene mutations in type I ovarian cancer has yet to be elucidated fully.

¹Department of Obstetrics and Gynecology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, 951-8510, Japan. ²Human Genetics Laboratory, National Institute of Genetics, Mishima, 411-8540, Japan. ³Department of Molecular and Diagnostic Pathology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, 951-8510, Japan. ⁴These authors contributed equally: Kentaro Sugino, Ryo Tamura and Hirofumi Nakaoka. *email: yoshikou@med.niigata-u.ac.jp
BRCA1/2 mutations. Of them, five patients were diagnosed with HGSC harboring germline second-degree relatives, 6 of 22 patients (27%) with a germline HR-associated gene mutation had family history. We identified one patient each of CCC, EC, and MC. When we focused on ovarian or breast cancer history in first- or second-degree relatives, 22 ovarian cancer patients. Among the 24 germline HR-associated gene mutations, 12 (50%) were detected in HGSC patients, and almost all of them were either BRCA2 mutation was identified in one patient each of CCC, EC, and MC. When we focused on ovarian or breast cancer history in first- or second-degree relatives, 6 of 22 patients (27%) with a germline HR-associated gene mutation had family history. Of them, five patients were diagnosed with HGSC harboring germline BRCA1/2 mutations.

In this study, we focused on difference in the distribution of ovarian cancer histologic subtypes between Western countries and Japan. For example, clear cell carcinoma (CCC) accounts for 25% of ovarian cancer in Japan and less than 10% in the United States. Therefore, we aimed to identify the frequency of germline and somatic HR-associated gene mutations per major histological subtypes of ovarian cancer in Japan, suggesting the therapeutic strategy of PARP inhibitors for ovarian cancers with HR-associated gene mutations.

### Results

#### Clinicopathological characteristics of ovarian cancer patients.

Clinicopathological characteristics of 207 patients (197 ovarian, 9 peritoneal, and 1 fallopian tube cancer) are shown in Table 1. Neoadjuvant chemotherapy cases that mainly consisted of HGSC patients were excluded. The median onset age of all patients was 56.0 years and more than half of patients (66%) were diagnosed at stage I. In all patients, the frequency of CCC (48%) was higher than that of HGSC (24%) that was the most common type of epithelial ovarian carcinoma. Although more than half of HGSC and low-grade serous carcinoma (LGSC) patients were at stage III, over 60% of CCC, endometrioid carcinoma (EC), and mucinous carcinoma (MC) patients were at stage I.

Next, we assessed mutation status of five genes (TP53, ARID1A, PIK3CA, KRAS, and PTEN), which were frequently mutated in ovarian cancer, for each histologic type (Table 1). Most of HGSC (82%) and MC (62%) patients harbored TP53 somatic mutations. CCC patients were characterized by high frequency of ARID1A (70%) and PIK3CA (64%) somatic mutations. EC patients harbored KRAS (46%) and PTEN (39%) somatic mutations in addition to ARID1A (46%) and PIK3CA (41%) somatic mutations.

#### Landscape of HR-associated gene mutations in ovarian cancer.

We investigated germline and somatic mutations of 16 HR-associated genes in 207 ovarian cancer samples. The average sequencing depth and the percentage of the target lesion that covered at least 20 reads were on average 98.6 and 98.9% in all samples, respectively. All the somatic mutations in HR-associated genes are listed in Supplementary Table S2. Missense mutation was the most frequent type of mutation (64%), followed by stopgain mutation (21%), frameshift insertion and deletion (11%), and splicing mutation (3%). Among 207 samples, 42 samples (20%) harbored at least one HR-associated gene mutation. The frequencies of germline and somatic HR-associated mutations in each histological subtype are shown in Fig. 1. Germline or somatic HR-associated gene mutations were detected in 44% of HGSC, 28% of CCC, 23% of EC, and 16% of MC, and 17% of LGSC patients. We investigated the correlation between stage and HR-associated gene mutations in each histological subtype (HGSC, CCC, and EC). All ECs harboring HR-associated gene mutations or germline BRCA mutations were diagnosed as stage I. On the other hand, there was no obvious difference of HR-associated mutation frequency per stage in HGSC and CCC (Supplementary Fig. S1).

All germline mutations identified in our dataset are shown in Table 2. We detected 24 germline mutations in 22 ovarian cancer patients. Among the 24 germline HR-associated gene mutations, 12 (50%) were detected in HGSC patients, and almost all of them were either BRCA1 or BRCA2. Intriguingly, BRCA2 mutation was identified in one patient each of CCC, EC, and MC. When we focused on ovarian or breast cancer history in first- or second-degree relatives, 6 of 22 patients (27%) with a germline HR-associated gene mutation had family history with ovarian cancer or breast cancer. Of them, five patients were diagnosed with HGSC harboring germline BRCA1/2 mutations.

#### Somatic HR-associated gene mutations per histological subtype.

Next, we compared the frequency of HR-associated gene alterations among three major histological subtypes (HGSC, CCC, and EC) of ovarian cancer (Fig. 2). BRCA1/2 somatic mutations were detected more frequently in HGSC patients (12%) than in CCC (5%) or EC patients (5%). However, ATM somatic mutations were detected more frequently in CCC (9%) and EC patients (16%) than in HGSC patients (4%). Most of the other HR-associated gene mutations were detected in a small population of each histological subtype.
Figure 1. Frequency of HR-associated gene mutation based on histology. The frequency of HR-associated gene mutation based on histology is shown in each pie chart. The mutation data were classified into seven categories – germline BRCA mutation (gBRCA m), somatic BRCA mutation (sBRCA m), both germline and somatic BRCA mutation (bBRCA m), other germline gene mutation (gOther m), other somatic gene mutation (sOther m), other germline and somatic gene mutation (bOther m), and no mutation.

| Patient ID | Age | Stage | Histological subtype | Gene | Class | Refseq ID | Nucleotide change | Amino acid change | Breast cancer patients in family | Ovarian cancer patients in family |
|------------|-----|-------|----------------------|------|-------|-----------|------------------|-----------------|-----------------------------|---------------------------------|
| 13         | 46  | I     | HGSC                 | BRCA1| frameshift deletion | NM_007300 | c.2028_2029del | p.T676fs         | Yes                         | Yes                             |
| 184        | 59  | III   | HGSC                 | BRCA1| stopgain           | NM_007300 | c.C2800T    | p.Q934X          | —                           | —                               |
| 566        | 49  | III   | HGSC                 | BRCA1| splicing           | NM_007300 | c.212 + 2T > C| —               | —                           | Yes                             |
| 614        | 56  | III   | HGSC                 | BRCA1| frameshift deletion| NM_007300 | c.321delT   | p.F107fs         | —                           | —                               |
| 723        | 72  | III   | HGSC                 | BRCA1| stopgain           | NM_007300 | c.C2800T    | p.Q934X          | —                           | —                               |
| 843        | 60  | III   | HGSC                 | BRCA1| splicing           | NM_007300 | c.5341–1G > A| —               | —                           | Yes                             |
| 584        | 75  | III   | HGSC                 | BRCA2| frameshift deletion| NM_000059 | c.9127delG  | p.E3042fs        | —                           | —                               |
| 1075       | 56  | II    | HGSC                 | BRCA2| frameshift insertion| NM_000059 | c.845_846insTTTG | p.H282fs | —                           | —                               |
| 1300       | 57  | IV    | HGSC                 | BRCA2| stopgain           | NM_000059 | c.9076T    | p.Q3026X         | —                           | —                               |
| 1357       | 62  | III   | HGSC                 | BRCA2| stopgain           | NM_000059 | c.6952T    | p.R2318X         | —                           | —                               |
| 602        | 61  | II    | CCC                  | BRCA2| stopgain           | NM_000059 | c.9076T    | p.Q3026X         | —                           | —                               |
| 152        | 50  | I     | EC                   | BRCA2| frameshift deletion| NM_000059 | c.5718_5719del | p.N1906fs | —                           | —                               |
| 734        | 61  | III   | MC                   | BRCA2| frameshift deletion| NM_000059 | c.958delC  | p.L320fs         | —                           | —                               |
| 210        | 60  | III   | CCC                  | ATM  | frameshift deletion| NM_000051 | c.4799delT  | p.V1600fs        | —                           | —                               |
| 292        | 52  | I     | CCC                  | BRI1 | splicing           | NM_032043 | c.2098–1G > A| —               | —                           | —                               |
| 396        | 32  | I     | MC                   | CHEK1| frameshift deletion| NM_01244864| c.668delA  | p.E223fs         | —                           | —                               |
| 90         | 60  | III   | CCC                  | EMSY | Non-frameshift deletion| NM_001300943| c.3289_3291del | p.1097_1097del | —                           | —                               |
| 1133       | 59  | II    | HGSC                 | RAD51D| frameshift deletion| NM_002878 | c.271_272insT | p.K91fs   | —                           | —                               |
| 237        | 86  | III   | CCC                  | RAD51D| frameshift deletion| NM_002878 | c.271_272insTA | p.K91fs   | —                           | —                               |
| 390        | 68  | I     | CCC                  | RAD51D| frameshift insertion| NM_002878 | c.271_272insTA | p.K91fs   | —                           | —                               |
| 184        | 59  | III   | HGSC                 | RAD54L| frameshift deletion| NM_003579 | c.1961delG  | p.R654fs         | —                           | —                               |
| 90         | 60  | III   | CCC                  | RAD54L| stopgain           | NM_003579 | c.1092_1093insC | p.G364_R | —                           | —                               |
| 261        | 53  | III   | CCC                  | RAD54L| stopgain           | NM_003579 | c.1092_1093insC | p.G364_R | —                           | —                               |
| 994        | 68  | I     | LGSC                 | RAD54L| stopgain           | NM_003579 | c.1092_1093insC | p.G364_R | —                           | —                               |

Table 2. Germline variants and clinical features.
Clinical significance of HR-associated gene mutations. We divided HGSC, CCC, and EC into two subgroups based on the status of HR-associated gene mutation and compared clinical characteristics between two subgroups in each histological subtype (Supplementary Table S3). EC patients with HR-associated gene mutation had younger age of onset than those without HR-associated gene mutations. No significant prognostic difference was observed between patients with and without HR-associated gene mutation irrespective of histology (Fig. 3). When we focused on only BRCA1/2 mutations, there were no significant differences in progression-free or overall survival between patients with and without BRCA1/2 mutations (Supplementary Fig. S2).

Correlation between mismatch repair gene mutations and HR-associated gene mutations. We investigated germline and somatic mutations of four mismatch repair (MMR) genes (MLH1, MSH2, MSH6, and PMS2) in 207 ovarian cancer cases (Table 3). Ten patients (5%) harbored deleterious germline or somatic mutations in MMR genes. The frequency of MMR gene mutation was 2% (HGSC), 3% (CCC), and 10% (EC) and there was no significant difference in the frequency of MMR gene mutation among histological subtypes. Subsequently, we investigated the correlation between MMR and HR-associated gene mutations. More than half of HR-associated gene mutation and MMR gene mutation were somatic mutations (63.3% and 70%) (Supplementary Fig. S3A). Patients with germline or somatic MMR gene mutation exhibited significantly higher frequency of germline or somatic HR-associated gene mutations (p = 0.0072) (Supplementary Fig. S3B).

Discussion
In this study, we demonstrated the frequency of germline and somatic HR-associated gene mutations in Japanese patients with ovarian cancer. In Japan, three studies19–21 have clarified the frequency of germline BRCA1/2 mutation in ovarian cancer but not somatic BRCA1/2 mutation. Pennington et al.7 have assessed the frequency of germline and somatic HR-associated gene mutations in 390 ovarian cancer dataset largely composed of HGSC. The Cancer Genome Atlas (TCGA) network4 has performed exome sequencing and clarified the frequency of germline and somatic HR-associated gene mutations in 316 HGSC cases. These studies have demonstrated that HR-associated gene mutations might correlate with better prognosis of HGSC patients. On the other hand, there was no report to assess the frequency and clinical significance of germline and somatic HR-associated gene mutations in a large scale of non-HGSC cases. Therefore, we focused on difference in the frequency of germline and somatic HR-associated gene mutations among major histological types of ovarian cancer.

Major histological subtypes of ovarian cancer are characterized by several cancer-associated gene mutations22. For example, TP53 mutation was detected in 97% of HGSC patients4 and more than 50% of MC patients23,24. KRAS mutation was found in 50% of MC and 18% of LGSC25 but not in HGSC. Similarly, ARID1A and PIK3CA were frequently mutated in CCC and EC patients but not in HGSC26–28. Our result was almost consistent with those of previous studies. Although the distribution of ovarian cancer histological subtypes is different between Japan and Western countries, representative mutation profiles per histological subtype are similar beyond ethnicity.

Corresponding to previous studies6,7, our findings showed that germline and somatic BRCA1/2 mutations were frequently identified in HGSC patients. Somatic BRCA1/2 mutations were also identified in a part of CCC (5%) and EC (5%). Interestingly, germline BRCA2 mutation was identified in one sample each of CCC, EC, and MC. HR-associated gene mutations were also detected in HGSC, CCC, and EC samples24,29. Surprisingly, our data indicated that a small part of the MC patients harbored HR-associated gene mutations and thereby, PARP inhibitors might be a potent therapeutic alternative for these cancers. However, the sample size of MC patients in this
The frequency of somatic ATM mutation was higher in CCC and EC than in other histological subtypes. The ATM protein kinase plays an important role in DNA damage response. Genetic alterations of ATM were found in many cancers, such as colorectal cancer (10%), prostate cancer (8.8%), lung cancer (7.3%), and ovarian cancer (4.5%). The frequency of ATM mutation in CCC was previously reported to be 7% (3/48) [27]. ATM-deficient tumor cells, such as mantle cell lymphoma [33] and colorectal cancer [32] exhibited high sensitivity to the PARP inhibitor, olaparib both in vitro and in vivo [34]. In a phase 2 clinical trial to assess the effectiveness of olaparib for metastatic prostate cancer and gastric cancer, ATM-mutated cases had better prognosis than ATM-wildtype cases [35,36]. Therefore, further analysis will be needed to elucidate the clinical significance of HR-associated gene mutations in MC, which is usually refractory to conventional platinum-taxane chemotherapy [30].

Table 3. Germline and somatic mutations of MMR genes.

| Patient ID | Age | Stage | Histological subtype | Gene Class | Refseq ID | Nucleotide change | Amino acid change | HR-associated gene mutation |
|------------|-----|-------|----------------------|------------|-----------|------------------|-------------------|--------------------------|
| Germline   |     |       |                      |            |           |                  |                   |                          |
| 762        | 38  | I     | EC                   | MSH2       | NM_000251 | c.131_132insG    | p.T44fs           | sATM, sPALB2            |
| 509        | 47  | I     | EC                   | MSH6       | NM_001281492 | c.C583T         | p.Q195X            | sATM, sCHEK1            |
| 329        | 59  | II    | HGSC                 | MSH6       | NM_001281492 | c.3692_3693insG | p.X1231delinsX     | sBRCA1                  |
| 378        | 53  | II    | CCC                  | MLH1       | NM_000249  | c.C350T          | p.T117M           |                          |
| 519        | 37  | III   | CCC                  | MLH1       | NM_001167617 | c.C688G         | p.Q230E            | sATM                    |
| 555        | 50  | III   | HGSC                 | MLH1       | NM_000249  | c.T107C          | p.I36T             |                          |
| 646        | 46  | I     | EC                   | MLH1       | NM_000249  | c.A525C          | p.K175N            | sBRCA2, sATM, sRAD1, sCHEK1, sCHEK2, sNBN, sRAD50 |
| Somatic    |     |       |                      |            |           |                  |                   |                          |
| 762        | 38  | I     | EC                   | MSH2       | NM_000251 | c.1706delA       | p.E569fs           | sATM, sPALB2            |
| 1031       | 43  | I     | CCC                  | MSH2       | NM_001258281 | c.1403delG     | p.R468fs          |                          |
| 1146       | 50  | I     | EC                   | MSH2       | NM_000251  | c.67delT         | p.F23fs            | sATM, sFANC1            |
| 762        | 38  | I     | EC                   | MSH6       | NM_000179  | c.3254delC       | p.T1085fs          | sATM, sPALB2            |
| 509        | 47  | I     | EC                   | MSH6       | NM_001281492 | c.297delG     | p.E99fs            | sATM, sCHEK1            |
| 762        | 38  | I     | EC                   | PMS2       | NM_000535  | c.C1882T         | p.R628X            | sATM, sPALB2            |
| 1321       | 41  | I     | EC                   | PMS2       | NM_000535  | c.C637T          | p.P213S            | sBRCA2, sCHEK2, sRAD51B |

Figure 3. Association between HR-associated gene alterations and clinical outcome in HGSC, CCC, and EC. Kaplan–Meier estimates of progression-free survival (A) and overall survival (B) in HGSC, CC, and EC.
PARP inhibitors might provide a survival advantage to ATM-mutated ovarian cancer, especially for CCC and EC types and tumor sequencing might be important not to miss these somatic mutations.

Mismatch repair (MMR) is a repair system of base mismatch pairing caused in DNA replication, and MMR genes (MLH1, MSH2, MSH6, and PMS2) often react to errors of a DNA single strand break. MMR gene mutations lead to microsatellite instability (MSI), and loss of function in germline MMR gene alterations cause Lynch syndrome. PD-1 antibody contributes to a favorable benefit for patients with MSI-high cancer. Our results showed positive correlation between mutation status of HR-associated genes and mismatch repair genes. It is inconclusive that defective MMR might contribute to platinum sensitivity for HGSC. However, Fleury et al. have reported that downregulation of both HR pathway and MMR pathway increases sensitivity of PARP inhibitor.

Furthermore, the phase 2 study for advanced or metastatic triple-negative breast cancer has revealed that combination therapy of Niraparib with Pembrolizumab confers higher response rates in patients with tumor BRCA mutation. Therefore, clinical trials evaluating the efficacy of PARP inhibitor and PD-1 antibody combination should be conducted for ovarian cancer patients.

Our findings indicated the possibility of HRD in major histological types of ovarian cancer. When we divided each histological group into two subgroups on the basis of HR-associated gene status in this study, there were no significant differences in clinical outcome between the two subgroups. This might be due to several reasons. First, most of patients in this study were stage I. Second, each HR-associated gene mutation was not evaluated at protein level for its pathogenicity. At least, we might need to evaluate an association of HR-associated gene mutation with other HRD assessment such as HRD score and HRDetect. Third, the sample size of each histological subtype was still small, and we could not exclude any influence of other prognostic factors such as stage, residual disease, and chemotherapy.

In conclusion, we elucidated the mutation profile of HR-associated genes in major histological types of ovarian cancer. PARP inhibitors might provide survival advantage to ovarian cancer patients with HR-associated gene mutations beyond histological subtypes.

**Methods**

**Clinical specimens.** This study was performed in conformity with the Declaration of Helsinki and approved by the institutional ethics review board at Niigata University (G2016-0006). The subjects were 207 epithelial ovarian cancer patients who had undergone initial surgery between July 2006 and September 2017 at Niigata University Medical and Dental Hospital. During the study period, we had enrolled 376 epithelial ovarian cancer patients. At first, 41 cases receiving neoadjuvant chemotherapy were excluded from this study. Then, 128 cases that could not provide the required DNA quality for blood and/or tumor samples were excluded. Finally, 207 epithelial ovarian cancer samples were collected as a cohort. All patients provided written informed consent for sample collection and subsequent analysis. Fresh-frozen tissue samples were obtained from primary tumor tissues. Staging of ovarian cancer was done following the criteria of the International Federation of Gynecology and Obstetrics (FIGO). Histological diagnosis was performed by two gynecological pathologists belonging to the Japanese Society of Pathology and assessed on formalin-fixed and paraffin-embedded hematoxylin and eosin sections. Histological subtypes were diagnosed according to WHO classification of ovarian tumors. Tumor DNA was extracted from frozen tissues containing more than 70% of tumor cells, revealed by histological evaluation. Disease progression was defined as at least a 20% growth in the size of the tumor or the longest diameters of lesions or as the appearance of one or more new lesions and/or unequivocal progression existing non-target lesions. Disease progression was defined as at least a 20% growth in the size of the tumor or the longest diameters of lesions or as the appearance of one or more new lesions and/or unequivocal progression existing non-target lesions since primary surgery following standard Response Evaluation Criteria In Solid Tumors (RECIST) guidelines. Overall survival time was evaluated for the interval from primary surgery to the death by ovarian cancer.

**DNA extraction.** Tumor DNA extraction was performed with Tissue Genomic DNA Extraction Mini Kit (FAVORGEN), according to the manufacturer’s instructions. Blood DNA was extracted with the QiAamp DNA Blood Maxi kit (Qiagen) following the manufacturer’s instructions. Genomic DNA was quantified using a Qubit dsDNA HS Assay Kit (Thermo Scientific).

**Targeted sequencing.** Targeted sequencing of 16 HR-associated genes, 5 ovarian cancer-associated genes, and 4 mismatch repair genes (Supplementary Table S1) was conducted with the pre-capture pooling method described in our previous study. In summary, 20 ng of DNA was simultaneously fragmented and adapter-ligated with SureSelect QXT Library Prep Kit (Agilent Technologies). The fragmented libraries with distinct indexed adapters were preserved at equimolar amounts. Subsequently, target enrichment was performed using the SeqCap EZ Choice System (Roche Diagnostics). A DNA probe set complementary to the target region was selected by NimbleDesign (https://design.nimblegen.com). The libraries were sequenced on a MiSeq platform (illumina). The paired-end read data were aligned to a human reference genome (hg19) using BWA and SAMtools. The aligned reads were processed for removal of PCR duplicates using Picard tools (v.1.111; broadinstitute.github.io/picard). Local realignments and base-quality recalibrations were conducted using GATK (v.3.2.2)52. The averages of depth and the coverages were calculated by the DepthOfCoverage and CallableLoci tools in GATK. Somatic single nucleotide variants (SNVs) and short insertions and deletions (indels) were called in each pair of tumor and matched normal blood samples using Strelka (v.1.0.14) workflow software. Sites with a depth greater than or equal to 20 in both tumor and matching normal blood samples, were subjected to somatic variant calling. We set the quality-score threshold to greater than or equal to 30 for SNVs and 50 for indels. Functional annotation of the identified somatic variants was implemented by ANNOVAR. The prevalence of somatic mutations indicated in previous genome-wide screenings in various cancer types were collected from the COSMIC database. The detected variants in germline BRCA1/2 were interpreted using BRCA Exchange and confirmed that there was no pathogenic germline missense BRCA1/2 mutation. In germline mutation analysis, stopgain,
frameshift, and splicing mutations were used. In addition to these mutation types, non-frameshift indel and missense mutations were used in somatic mutation analysis.

**Statistical analysis.** All computations were conducted using R. Standard statistical tests were used as appropriate, including unpaired t-test, Welch’s exact test, and logrank test.

**Data availability**
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
K.Y. and T.E. designed the research plan. K.S., R.T., N.Y., M.Y., K.Y., K.S., T.I., S.A. and K.Y. collected tissue samples. H.N. and I.I. performed sequencing experiments. K.S. and R.T. conducted all analyses. K.S. and R.T. wrote the main manuscript and prepared all figures and tables. K.S., R.T., H.N., I.I., K.Y. and T.E. discussed the results. All authors reviewed and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to K.Y.
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