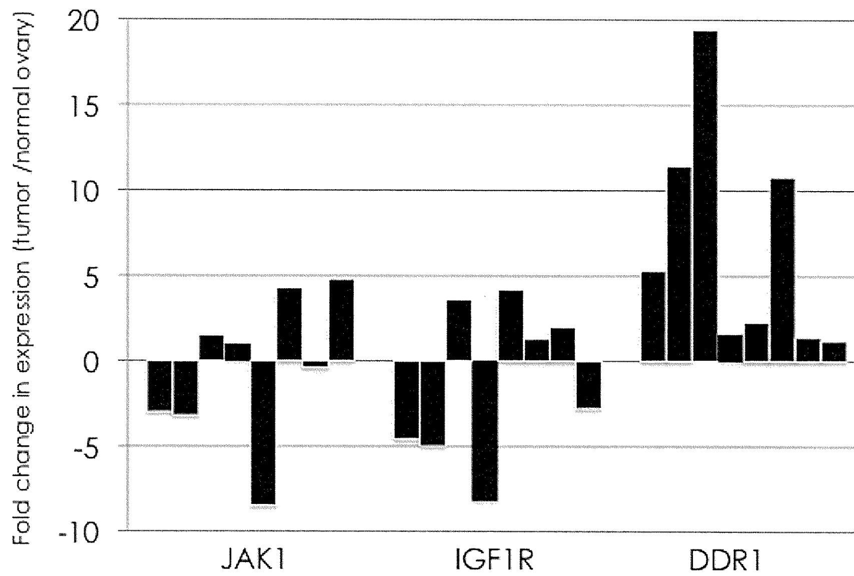
Figure 1. Quantitative real-time PCR for the validation of overexpression of three identified genes in ovarian cancer. Quantitative real-time PCR was performed to validate the overexpression of three identified PTKs, JAK1, IGF1R, and DDR1, in ovarian cancer. Fold change in the expression between ovarian cancer tissue and their corresponding normal ovarian tissue samples are shown with black bars. The DDR1 gene was overexpressed in all eight individuals, whereas the expression levels of JAK1 and IGF1-R were not increased in half of the ovarian cancer tissue samples.



To characterize the expression of DDR1, we carried out immunohistochemical analysis of 67 primary serous ovarian cancers from patients who had not received any prior treatment, obtained from primary debulking surgery. As demonstrated in Figure 2, no DDR1 staining was present in epithelial cells in normal ovary and serous adenoma, but the expression was increased in the ovarian cancer cells.

The DDR1 protein was highly expressed in 69% (46/67) of serous ovarian cancer tissue samples. Moreover, DDR1 protein expression was correlated with the pathologic grade of the tumor and the clinical disease stage at the time of surgery. As shown in Table 1, DDR1 was expressed significantly more frequently in FIGO Grade 1 (50%) compared to combined G2 and G3 (79%) tumors ($p = 0.015$).

We next compared the clinical disease stage for patients with different levels of DDR1 expression. Given the limited number of samples, the stage I and II samples were combined (early stage), as were the stage III and IV samples (advanced stage). The expression of DDR1 in early stage samples was 50%, while it was 82% in advanced stage tumors. There were significant differences in DDR1 expression found between advanced stage tumors compared with tumors found in the early stage ($p = 0.006$) (Table 2).

DDR1 expression was then examined for an association with disease-free survival and overall survival using Kaplan-Meier survival analysis with the log-rank statistic to determine significance. Kaplan-Meier survival curves generated for tumor DDR1, high *versus* low expression, are given in Figure 3. High tumor DDR1 expression was significantly associated with a poor outcome for disease-free survival ($p = 0.032$). With regard to overall survival, high tumor DDR1 expression showed a tendency toward a poorer outcome, but this trend was not statistically significant ($p = 0.064$).

Figure 2. Representative immunohistochemistry staining of DDR1. Immunohistochemical analysis with the DDR1 antibody revealed negative DDR1 protein expression in (**A**) normal ovary and (**B**) serous cystadenoma, whereas positive expression was observed in (**C**) serous ovarian cancer cells. (**A**) no DDR1 staining in surface epithelium of normal ovary (black arrow); (**B**) no DDR1 staining in epithelial lining cells in serous cystadenoma (black arrow); (**C**) representative staining pattern of serous ovarian cancer tissue shows positive DDR1 staining in most serous adenocarcinoma cells (black arrow) with negative DDR1 staining in stromal cells (white arrow); (**D**) in placenta (positive control); all trophoblastic cells show positive DDR1 staining. Original magnification: ×40, scale bar: 50 μm (white bar).

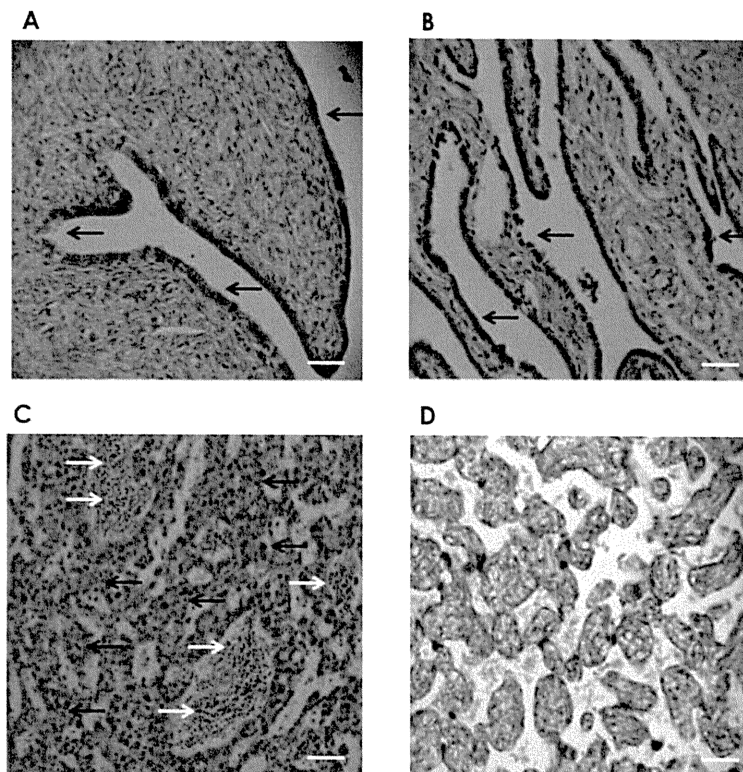


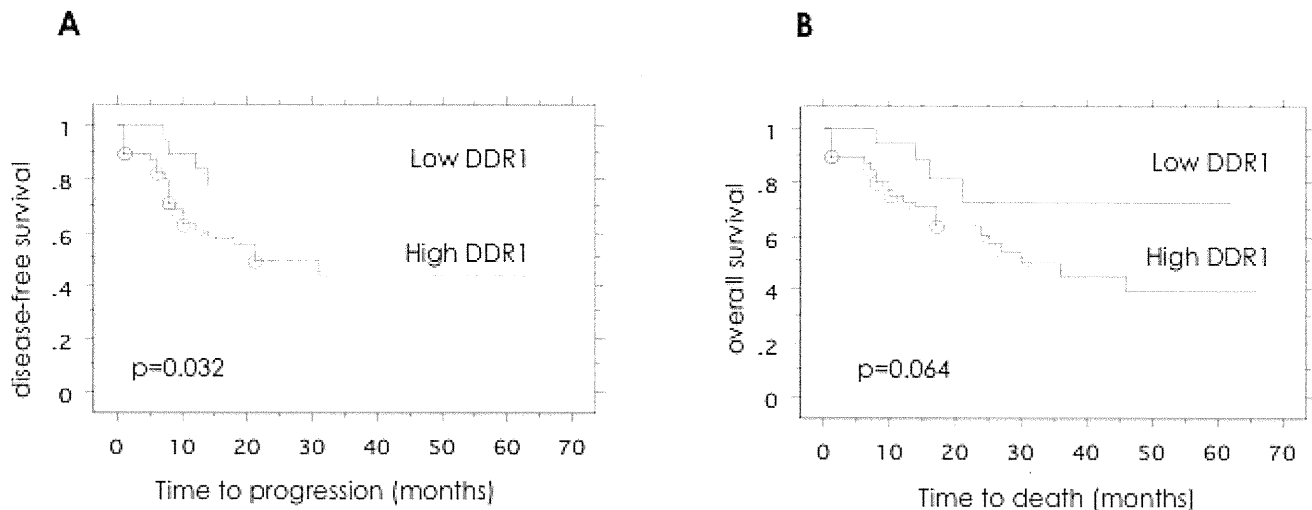
Table 1. DDR1 expression in patients with ovarian cancer according to tumor grading.

	DDR1 positive	DDR1 negative	
G1	12	12	
G2	21	5	$P = 0.015$
G3	13	4	

Table 2. DDR1 expression in patients with ovarian cancer according to clinical staging.

Stage	Cases No.	DDR1 positive	Percent of DDR1 positive	
I	22	11	50.0	$p = 0.006$
II	6	3		
III	35	29	82.1	
IV	4	3		
total	67	46	68.7	

Figure 3. Patient outcome according to the DDR1 expression in patients with serous ovarian cancer. Kaplan-Meier analysis of (A) disease-free survival and (B) overall survival, according to DDR1 expression levels. Significant trend for shorter disease-free survival was observed in the DDR1 positive group ($p = 0.043$).



4. Discussion

In the present study, we identified DDR1 as a differentially expressed PTK gene in primary epithelial serous ovarian cancer using a combination of cDNA subtraction and degenerate PCR-based cloning. DDR1 was more highly expressed in ovarian cancer samples compared with normal ovarian tissues. We were able to show that DDR1 expression is associated with the tumor grade and clinical disease stage, and is inversely correlated with the survival outcome of patients.

Receptor tyrosine kinases control a wide array of cellular responses, including the regulation of cell growth, differentiation, migration, metabolism, and survival. DDR1 was independently isolated as a novel receptor tyrosine kinase by several laboratories from human, mouse, and rat tissue in the 1990s [9–11]. DDR1 is characterized by a structural domain of 160 amino acids in its extracellular part that exhibits strong sequence similarity to the *Dictyostelium discoideum* protein discoidin 1, coagulation factors V and VIII, and to a *Xenopus laevis* recognition protein, A5. DDR1 is activated by collagen type I, II, III, V, and XI. Activation of DDR1 by collagen results in its sustained intracellular phosphorylation. DDR1 is widely expressed in epithelial cells of both fetal and adult organs. Although the physiological

functions of DDR1 are not fully understood, DDR1 signaling is essential for cerebellar granule differentiation [12], arterial wound repair [13], and mammary gland development [14]. It is clear that DDR1 is involved in cell interactions with the extracellular matrix, and that it controls adhesion and cell motility [15,16].

DDR1 was found to be overexpressed in breast, brain, colon, and lung cancers, thus suggesting that this receptor may play a role in the tumorigenesis of epithelial cancers [17–20]. In breast cancer, DDR1 was overexpressed in both primary breast tumor samples and metastasis-containing lymph nodes [21]. DDR1 protein levels were elevated in 100% of patients with primary and metastatic brain tumors [18], in 61% of patients with non-small cell lung cancer, and in 64% of patients with invasive lung adenocarcinoma [22]. Thus, DDR1 expression appears to be elevated in a variety of human cancers. Consistent with studies of DDR1 in these solid tumors, elevated levels of DDR1 were seen in serous ovarian cancer in this study. Our results were also consistent with a study by Heinzelmann-Schwarz *et al.* that reported that DDR1 proteins are highly overexpressed in all histological subtypes of epithelial ovarian cancer compared with the normal ovarian surface epithelium [23].

The overexpression of DDR1 in these different human cancers suggests that it may have a function in tumor progression. It has been reported that DDR1 is overexpressed in high-grade brain, esophageal, and breast cancers, and high expression of DDR1 was associated with a significantly poorer survival in several cohorts of patients with brain, breast, and lung cancers [18,22,24–26]. In the present study, we showed that DDR1 expression was associated with high-grade and advanced stage tumors, as well as with poor survival in patients with ovarian cancer. These results were not consistent with those from previous study by Heinzelmann-Schwarz *et al.* [23]. They reported that expression of membranous DDR1 did not correlate with survival of patients. Recently Mihai C. *et al.* proposed the model of the DDR1 activation mechanism by analyzing the cellular distribution of DDR1 [27]. They showed the aggregation and cellular internalization of the receptor following collagen stimulation. In this study, we evaluated both membranous and cytoplasmic DDR1 expression and did not analyze the intensity of the staining. Although the activation process of DDR1 still remains largely unknown, evaluation of the cellular distribution or intensity of DDR1 expression might be required to analyze the precise biological activity of DDR1. Several recent studies have examined the molecular mechanisms underlying the role of DDR1 in tumor progression, invasion, and metastasis. One study showed that DDR1 expression may be regulated during the cell cycle, because overexpression of p53 in osteosarcoma cells induces DDR1 expression [28]. Ongusaha *et al.* have reported that DDR1 is a direct p53 transcriptional target, and that inhibition of DDR1 function resulted in increased apoptosis through a caspase-dependent pathway [29]. Other published data imply that the Wnt-5a pathway may overlap with DDR1 signaling [26]. Shintani *et al.* have shown that DDR1 promotes the epithelial to mesenchymal transition in response to collagen I stimulation in human pancreatic cancer cells [30]. Although experimental evidence argues against a classification of DDR1 as a transforming oncogene, subsequent steps after the initial cellular transformation, such as invasion and metastasis, might be mediated by DDR1.

One of the major characteristics of an ideal biomarker or target for molecular therapeutics is that it is absent in benign tissue and present in the targeted malignancies. Our results showed no protein expression of DDR1 in normal ovarian epithelial cells. It has been reported that DDR1 expression is highest in the brain, lungs, placenta and kidneys, and is present at low levels in various other adult

tissues, such as melanocytes, the heart, liver, skeletal muscle, pancreas, and ovaries [31]. Elevated levels of DDR1 protein expression appear to be highly predictive of the presence of ovarian cancer. Although it would be nonspecific for ovarian cancer (because it could also indicate various other malignancies), the levels of DDR1 expression in body fluid or serum may have clinical prognostic utility as a biomarker for cancer.

Various targeted therapeutics have been explored for the management of ovarian cancer. In addition, numerous studies have examined the use of TKIs, including monoclonal antibodies against Her2/neu [4], other EGFRs [3], and VEGF [5], and small molecule tyrosine kinase inhibitors targeting various other receptors, including the EGFR and VEGFR [32,33]. A recent study showed that dasatinib, a multi-targeted TKI, inhibits DDR1, in addition to inhibiting BCR-ABL [34]. Moreover, DDR1 has also been identified as a potential target of other BCR-ABL inhibitors, including imatinib [35]. These inhibitors of DDR1 may prove to be therapeutically beneficial for the treatment of advanced ovarian cancer.

5. Conclusion

In summary, we have identified DDR1 as a differentially overexpressed PTK in ovarian cancer tissue using a combination of cDNA subtraction and degenerate PCR-based cloning. The association between DDR1 expression, tumor grade, clinical disease stage, and patient outcome suggests an *in vivo* role for this signal transduction pathway in ovarian cancer. The mechanisms by which DDR1 affects signaling cascades involved in tumor progression, invasion, and metastasis have not yet been fully characterized. Further investigation of DDR1 as a clinical biomarker and as a therapeutic target is warranted, especially for ovarian cancer.

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Germline Copy Number Variations in *BRCA1*-Associated Ovarian Cancer Patients

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We investigated characteristics of germline copy number variations (CNV) in *BRCA1*-associated ovarian cancer patients by comparing them to CNVs present in sporadic ovarian cancer patients. Germline CNVs in 51 *BRCA1*-associated, 33 sporadic ovarian cancer patients, and 47 healthy women were analyzed by both signal intensity and genotyping data using the Affymetrix Genome-Wide Human SNP Array 6.0. The total number of CNVs per genome was greater in the sporadic group (median 26, range 12–34) than in the *BRCA1* group (median 21, range 11–35; *post hoc* $P < 0.05$) or normal group (median 20, range 7–32; *post hoc* $P < 0.05$). While the number of amplifications per genome was higher in the sporadic group (median 13, range 7–26) than in the *BRCA1* group (median 8, range 3–23; *post hoc* $P < 0.001$), the number of deletions per genome was higher in the *BRCA1* group (median 12, range 6–24) than in the sporadic group (median 9, range 3–17; *post hoc* $P < 0.01$). In addition, 31 previously unknown CNV regions were present specifically in the *BRCA1* group. When we performed pathway analysis on the 241 overlapping genes mapped to these novel CNV regions, the 'purine metabolism' and '14-3-3-mediated signaling' pathways were over-represented (Fisher's exact test, $P < 0.01$). Our study shows that there are qualitative differences in genomic CNV profiles between *BRCA1*-associated and sporadic ovarian cancer patients. Further studies are necessary to clarify the significance of the genomic CNV profile unique to *BRCA1*-associated ovarian cancer patients. © 2010 Wiley-Liss, Inc.

INTRODUCTION

A copy number variation (CNV) is a segment of DNA 1 kb or larger that is present at variable copy numbers in comparison to a reference genome (Feuk et al., 2006). Recently, germline CNVs have been recognized not only as causes of rare genetic disorders but also as important susceptibility factors for a range of common diseases including infectious, autoimmune, and neuropsychiatric diseases (Cohen, 2007; Wain et al., 2009; Zhang et al., 2009; Fanciulli et al., 2010). In the field of cancer research, somatic CNVs are detected in the genomes of various cancer cells, and some of them are thought to play an important role in carcinogenesis (Shlien and Malkin, 2009; Fanciulli et al., 2010). Shlien et al. (2008) reported excessive genomic CNVs in Li-Fraumeni syndrome (LFS), which is an autosomal dominant disorder characterized by increased risk of various cancers in individuals with germline *TP53* mutations. However, the role of germline CNVs in individuals predisposed to cancer or in cancer patients has not yet been clarified.

Familial ovarian cancer accounts for 5%–10% of all epithelial ovarian cancers (Whittemore et al., 1997), and most familial cases are associated with mutations in Breast cancer 1, early onset (*BRCA1*; Easton et al., 1995). *BRCA1* is located on chromosome 17q21 and plays critical roles in DNA repair, cell cycle checkpoint control, and maintenance of genomic stability (Gudmundsdottir and Ashworth, 2006). Histologically, most *BRCA1*-associated ovarian cancers are serous papillary adenocarcinomas (Sekine et al., 2001). Interestingly, the genomic profile for *BRCA1*-associated ovarian cancer is distinct from that for sporadic ovarian cancer, although they have the same histological type (Patael-Karasik et al., 2000; Zweemer et al., 2001; Israeli et al., 2003; Walsh et al., 2008). Therefore, it is thought that *BRCA1*-

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associated and sporadic serous ovarian cancers arise from different molecular pathways (Walsh et al., 2008). To date, studies regarding differences in germline CNVs between *BRCA1*-associated and sporadic ovarian cancers have been not reported.

Previously, we analyzed genetic alterations in *BRCA1* among Japanese ovarian cancer families, demonstrating the clinicopathological characteristics of *BRCA1*-associated ovarian cancer patients. In the present study, we sought to identify the genome-wide profile of germline CNVs in *BRCA1*-associated ovarian cancers using an ultra-high-resolution single nucleotide polymorphism (SNP) array and to elucidate the molecular characteristics of germline CNVs in *BRCA1*-associated ovarian cancer patients, when compared to sporadic ovarian cancer patients or healthy women.

MATERIALS AND METHODS

Subjects

In this study, we recruited 71 individuals with germline *BRCA1* mutations, 47 healthy controls aged >65 years with no history of cancer, and 34 sporadic ovarian cancer patients without any familial history of cancer. Patients and controls were all of Japanese ethnicity. The ethics committees of the participating institutions approved the study protocol and each participant gave written informed consent. Germline mutations in *BRCA1* were examined according to an in-house protocol (Sekine et al., 2001). Genomic DNA was prepared from lymphocytes using the standard phenol-chloroform method.

SNP Array Experiments

For analysis with the Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA), genomic DNA preparation and chip processing were performed according to the Affymetrix recommended protocols. Briefly, 250 ng of genomic DNA was digested with either *Nsp*I or *Sty*I (New England Biolabs, Inc., Ipswich, MA) and then ligated to Nsp or Sty adaptors, respectively. The adaptor-ligated DNA fragments were amplified by polymerase chain reaction (PCR) using a single primer that recognized the adaptor sequence. All products from each sample were combined and purified using magnetic beads (Agencourt AMPure, Beckman Coulter, Beverly, MA). The purified PCR products were fragmented using DNase I, end-labeled with a biotinylated nucleotide, and hybridized to a Genome-Wide Human SNP Array 6.0 (Affymetrix) at 50°C for 17 hr. After hybridization, the arrays were washed, stained, and scanned with a GeneChip Scanner 3000 7G (Affymetrix).

Data Analysis

Quality control (QC) was performed using Genotyping Console 4.0 (Affymetrix). The median of the absolute values of all pairwise differences between $\log_2$ ratios for a given chip (MAPD) was calculated for all samples. Samples with a MAPD value greater than 0.30 were not included in the study. The QC call rate of recruited samples was higher than 90%. After sample QC, chip data from 68 *BRCA1* mutation carriers (51 affected carriers and 17 carriers without cancer), 47 healthy controls, and 33 sporadic ovarian cancer patients were used in the subsequent analysis.

We prepared a custom reference panel for array-based CNV analysis. Although 270 HapMap samples have been used in many reports for copy number analysis (Redon et al., 2006; McCarroll et al., 2008), the 270 HapMap samples are from multiethnic populations and derived from lymphoblastoid cell lines. Previously, it was shown that there are variations in germline CNV distribution among different ethnic populations (White et al., 2007; Jakobsson et al., 2008; Li et al., 2009). Therefore, we selected SNP Array 6.0 data obtained from 330 Japanese women (Adachi et al., in press) as a reference, based on Affymetrix recommendations.

Data were processed using Partek Genomics Suite 6.5 (Partek Inc., St. Louis, MO). Genotype calls for the SNP probes were determined using the Birdseed v2 algorithm (Korn et al., 2008; Nishida et al., 2008). SNP call rates were higher than 97% in all samples. Regions of copy number alterations were detected using a Hidden Markov Model algorithm with the following parameters: maximum probability = 0.98, genomic decay = 1,000,000, and sigma = 1 (Walter et al., 2009). Because the median intermarker distance for the Genome-Wide Human SNP Array 6.0 is 680 bp, a CNV was defined as a change in the inferred copy number state in a genomic region covering at least five consecutive probes to avoid detecting false positive CNVs (Pinto et al., 2010). Gene annotation and overlap was determined using the University of California, Santa Cruz (UCSC) genome assembly (hg18) (http://genome.ucsc.edu/).

Detected CNVs in *BRCA1*-associated sporadic ovarian cancer patients and healthy controls were evaluated in terms of frequency and length. When we compared the frequency or length of CNV regions among *BRCA1*-associated sporadic ovarian cancer patients and healthy controls, the Kruskal-Wallis test was performed using GraphPad PRISM version 4.0 (GraphPad Software, San Diego, California, USA). When the Kruskal-Wallis test showed a significant difference, Dunn's multiple comparison test was performed as a *post hoc* test. *Post hoc* $P < 0.05$ was considered statistically significant. In addition, we used the Spearman rank correlation to measure the association between the number and length of CNVs per individual or between the number of CNVs and onset age at diagnosis using GraphPad PRISM version 4.0.

Genome CNV profiles measured by SNP Array 6.0 were compared between *BRCA1*-associated and sporadic ovarian cancer patients. Based on genome-wide CNV data for individuals, hierarchical clustering analysis was performed using a complete linkage clustering algorithm (Partek Genomic Suite 6.5) with Pearson correlation coefficients in all pairwise combinations (Haverty et al., 2009).

To investigate the biological characteristics of overlapping genes in detected CNV regions, Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com/) was used (Cuscó et al., 2009; Yoshihara et al., 2010). We performed 'Core Analysis' to examine which molecular function categories or pathways were statistically over-represented among the obtained gene list. Statistical significance was determined by Fisher's exact test using all annotated genes in IPA as a background.

RESULTS

Germline *BRCA1* Mutations

In this study, we evaluated 68 *BRCA1* mutation carriers in 41 *BRCA1* families. Of the 68 carriers, 51 were affected with ovarian cancer, and the median age at diagnosis was 52 years (range, 28–74 years). Histological analysis of the 51 patients with ovarian cancer showed that 43 (84.3%) were of the serous type, 6 (11.8%) were of the endometrioid type, and 2 (3.9%) were undifferentiated. The types of germline mutations in *BRCA1* are listed in Supporting Information Table 1. Nineteen *BRCA1* families were assigned to carriers with two types of nonsense mutations, L63X and Q934X, both of which are mutations specific to Japanese ovarian cancer patients (Sekine et al., 2001).

Identification of Germline CNVs

Germline CNVs in all samples were analyzed according to both signal intensity and genotyping data using the Affymetrix Genome-Wide Human SNP Array 6.0. To clarify the molecular characteristics of germline CNVs in *BRCA1*-associated ovarian cancer patients, we compared genome-wide profiles of germline CNVs among 51 *BRCA1* mutation carriers affected with ovarian cancer (*BRCA1* group), 33 sporadic ovarian cancer patients (sporadic group), and 47 healthy controls (normal group). The median ages were 52 years (range, 28–74) in the *BRCA1* group, 59 years (range, 34–81) in the sporadic group, and 73 years (range, 65–83) in the normal group.

Figure 1 shows a genome-wide frequency plot of germline CNVs in each group. Recurrent genomic changes among samples were observed at several chromosomal intervals. Most notably, CNVs at 1q21.3, 2q22.3, 4p16.1, 7q34, and 15q11.2 were observed in greater than 40% of each group. These CNVs had been previously reported in more than ten publications and submitted to the Database of Genomic Variants (http://projects.tcag.ca/variation/).

To evaluate the differences in CNV frequencies between the three groups, a Kruskal-Wallis test was performed. The total number of CNVs per genome was higher in the sporadic group (median 26, range 12–34) than in the *BRCA1* (median 21, range 11–35; *post hoc* $P < 0.05$) or normal group (median 20, range 7–32; *post hoc* $P < 0.05$; Fig. 2). Regarding the number of amplification regions, there were significant differences between the sporadic group (median 13, range 7–26) and the other two groups (*BRCA1* group: median 8, range 3–23; *post hoc* $P < 0.001$; normal group: median 9, range 1–20; *post hoc* $P < 0.001$). On the other hand, the number of deletion regions in the *BRCA1* group was higher than in the sporadic group (*post hoc* $P < 0.01$). The deletion/amplification ratio, which is, the per-sample ratio of the number of deletion regions to the number of amplification regions, yielded a median of 1.50 (range 0.50–7.00) in the *BRCA1* group, 1.30 (range 0.40–6.00) in the normal group, and 0.60 (range 0.30–1.70) in the sporadic group. In addition, we analyzed the differences in the lengths of CNV regions per individual among the three groups. Although the lengths of total CNVs per genome or amplification regions were

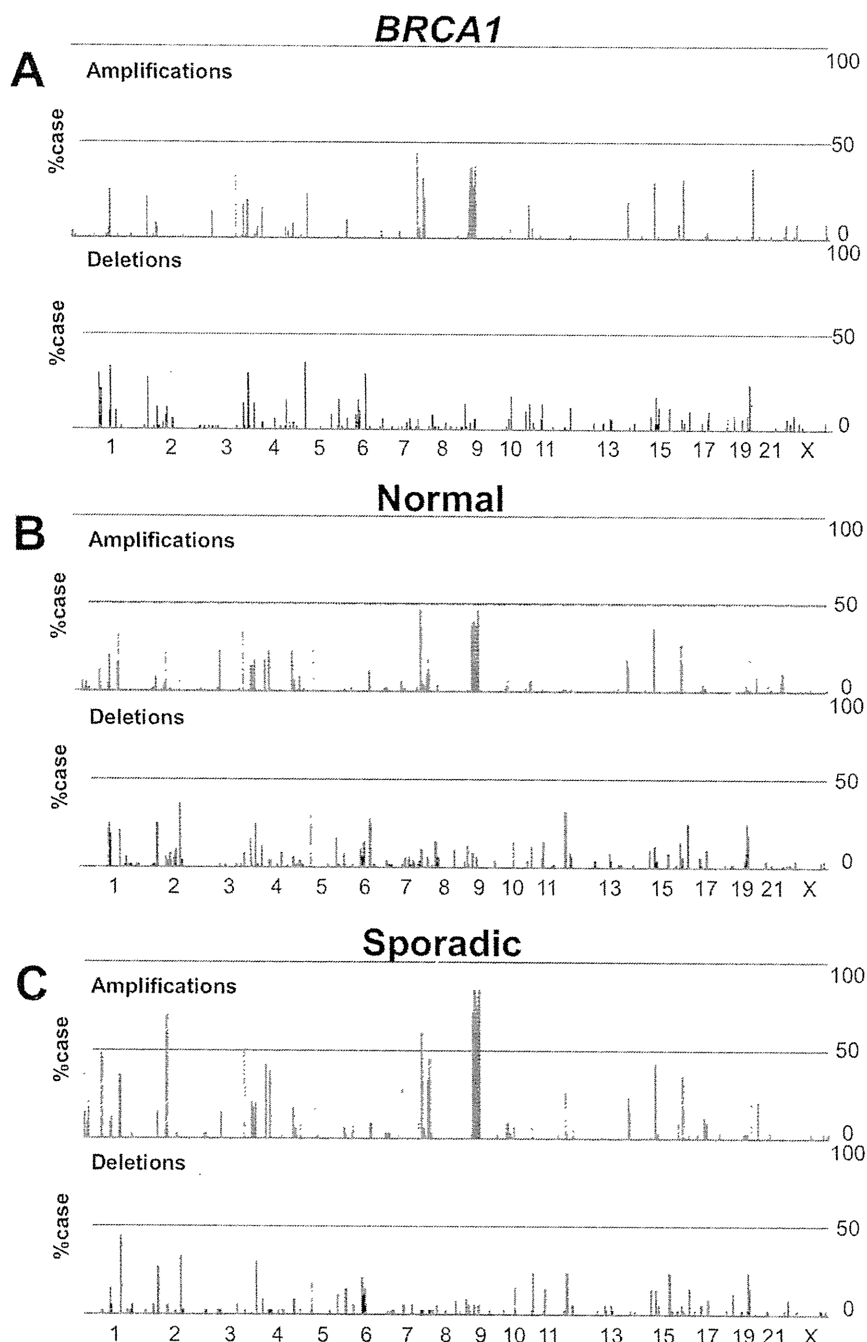

Figure 1. Frequency plot summarizing germline CNVs in *BRCA1*, sporadic, and normal groups. Amplifications are shown in red and deletions in blue. The genomic location is represented along the *x*-axis, with chromosome 1 on the left and the X chromosome on the right. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly greater in the sporadic group than in the *BRCA1* (total CNV, *post hoc* $P < 0.001$; amplification, *post hoc* $P < 0.001$) or normal groups (total CNV, *post hoc* $P < 0.001$; amplification, *post hoc* $P < 0.001$), there was no difference in the total length of the deletion regions among the three groups (Kruskal-Wallis test, $P = 0.55$; Supporting Information Fig. 1). Spearman's correlation analysis for all samples indicated that there was a significant correlation between the total number and total length of CNV regions (r: 0.47, $P < 0.0001$).

We focused our attention on small-scale CNVs, defined as segment sizes less than 50 kb, which were detected by new ultra-high-resolution techniques, because these small-scale CNVs could

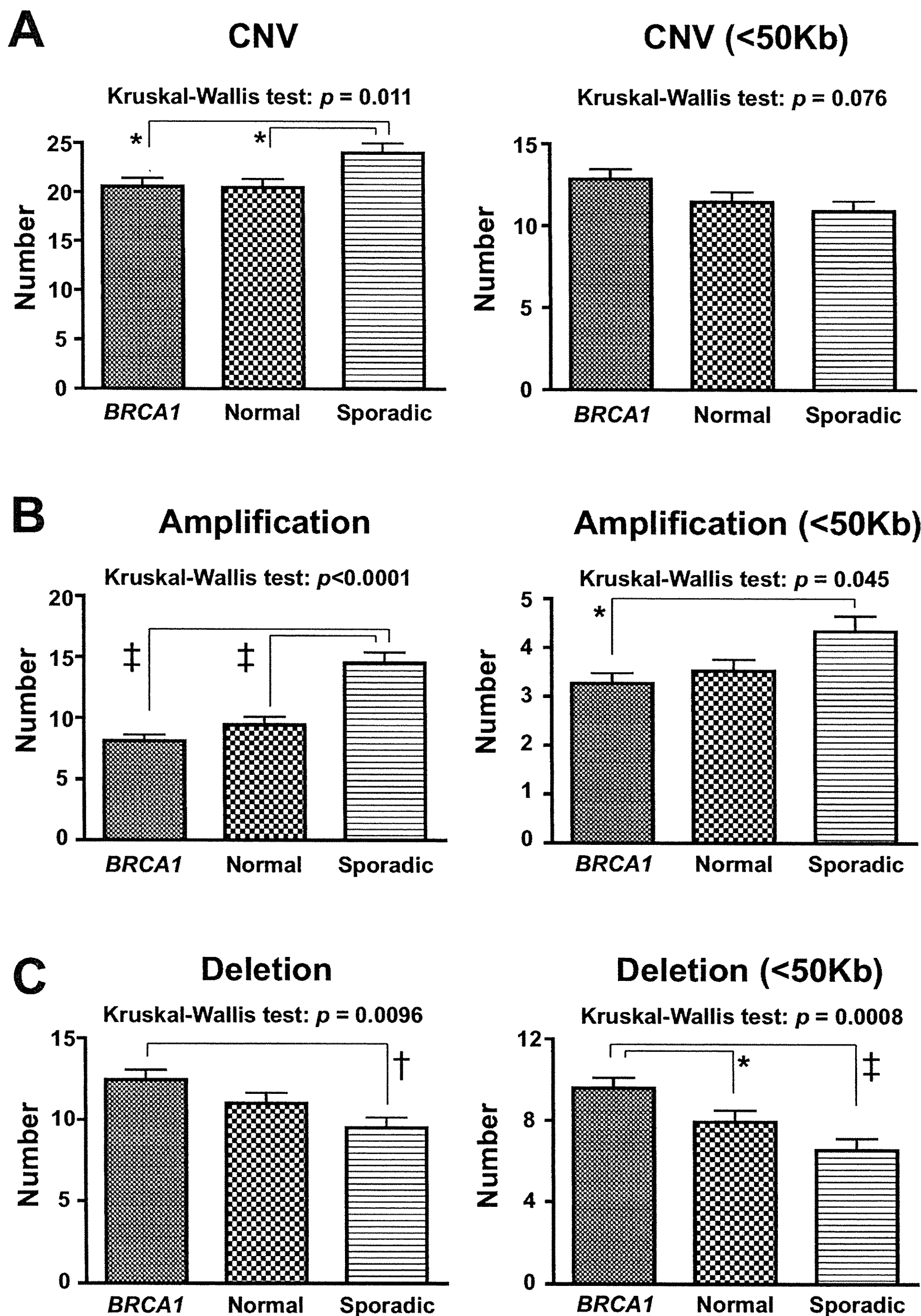

Figure 2. Differences in CNV frequency among *BRCA1*, sporadic, and normal groups. There are significant differences in total CNV number (A), amplification (B), and deletion regions (C) among the three groups. The cutoffs for *post hoc P* values in Dunn's multiple comparison test were set to 0.05 (*), 0.01 (†), and 0.001 (‡). When small-scale CNVs are defined as less than 50 kb, the results for the number of small-scale CNVs (A), amplification (B), and deletion regions are indicated in the right column.

not have been analyzed by previous methods such as clone-based array comparative genome hybridization or fluorescence *in situ* hybridization (Tuzun et al., 2005; Feuk et al., 2006). Interestingly, the number of small CNV regions in the *BRCA1* group was marginally higher than in the other two groups ($P = 0.076$). The *BRCA1* group had higher frequencies of small deletion regions than the other groups (sporadic, *post hoc* $P < 0.001$; normal, *post hoc* $P < 0.05$; Fig. 2).

Because each group displayed several features concerning the frequencies or lengths of CNV regions per individual, we next assessed whether there were differences in genomic CNV profiles between the three groups. We performed hierarchical clustering analysis using genome-wide CNV data for individuals. In this clustering analysis, we included 17 *BRCA1* mutation carriers who did not have ovarian cancer to investigate differences in genomic CNV profiles between carriers affected and not affected with ovarian cancer. However, we could not identify any clear clustering patterns that divided each group. Hierarchical clustering analysis could not discriminate between affected and unaffected carriers (Supporting Information Fig. 2). Indeed, there were no significant differences in CNV frequencies between 51 *BRCA1* mutation carriers affected with ovarian cancer and 17 unaffected carriers (data not shown).

It is known that there is inter-individual variability in the age of onset for ovarian cancer in *BRCA1* patients (Sekine et al., 2001; Sugano et al., 2008). Our study showed no significant linear correlation between age at diagnosis and CNV frequency (data not shown). When we compared five *BRCA1* carriers afflicted with ovarian cancer before age 40 (early-onset) to six *BRCA1* carriers not affected before age 65 (including three cases afflicted with ovarian cancer after age 65), there was no significant difference in CNV frequency between the two groups (data not shown).

BRCA1-Unique CNVs

To clarify the qualitative differences in genomic CNV profiles between *BRCA1*-associated and sporadic ovarian cancer patients, we investigated unique CNVs detected only in the *BRCA1* group compared to the normal and sporadic groups. In total, 111 CNVs were detected only in the *BRCA1* group. Furthermore, we selected novel CNVs that had not been registered in the Database of Genomic Variants (http://projects.tcag.ca/variation) as *BRCA1*-unique CNVs, according to the criteria that two CNVs are the same when they overlap for more than 70% of their predicted genomic regions (Cuscó et al., 2009). As a result, we found 31 *BRCA1*-unique CNV regions, all of which include at least one gene (Table 1). Of these 31 CNV regions, 3 CNVs on 1q43, Xp22.2-21.1, and Xp21.3 were shared among ovarian cancer patients (sisters or mother and daughter) from the same family. However, because samples from both parents of *BRCA1* mutation carriers were not available, we could not evaluate whether other CNV regions arose *de novo* or were the result of inherited genomic changes. In a similar way, 25 and 13 regions were detected as sporadic ovarian cancer-unique CNVs and normal-unique CNVs, respectively (Supporting Information Tables 2 and 3).

To elucidate the biological characteristics of the overlapping genes included in *BRCA1*-unique or sporadic-unique CNV regions, we used the 'Core Analysis' embedded in IPA software. Because we had excluded miscRNAs or pseudogenes from this analysis, 241 *BRCA1*-unique, 129 sporadic-unique, and 37 normal-unique overlapping genes were uploaded to the IPA software. To characterize the gene list based on IPA classification of molecular and cellular functions, we examined which predefined categories were highly associated with group-unique overlapping genes using Fisher's exact test. When the P value cutoff was set to 0.001 for the purpose of clarifying differences among the three groups, three categories ('cellular development', 'cellular movement', and 'small molecular biochemistry') were over-represented in the *BRCA1* group (P = 0.00032, 0.00036, and 0.00079, respectively), and the 'cellular development' category was over-represented in the sporadic group ($P = 0.00056$; Supporting Information Table 4). Genes involved in 'cellular development' were notably enriched in both *BRCA1*- and sporadic-unique genes. On the other hand, there was no category associated with overlapping genes in normal-unique CNVs.

When we further investigated the association between overlapping genes in the *BRCA1*-unique CNVs and 'ingenuity canonical pathways' excluding 'disease-specific pathways', the 'purine metabolism' and '14-3-3-mediated signaling' pathways were significantly over-represented (Fisher's exact test, $P < 0.01$). Of 241 genes in *BRCA1*-unique CNVs, nine genes (*ENTPD8*, *ABCA2*, *GUCY1A3*, *ATP5D*, *ENTPD2*, *POLR2E*, *POLRMT*, *APRT*, *POLR2K*) were mapped to the 'purine metabolism' pathway that contains 439 genes, and five genes (*TRAF2*, *TP73*, *TUBB2C*, *PLCH2*, *PRKCZ*) were mapped to the '14-3-3-mediated signaling' pathway that contains 116 genes. In contrast, we could not identify any significant association between 'Ingenuity canonical pathways' and overlapping genes in sporadic ovarian cancer or normal-unique CNVs.

DISCUSSION

We investigated quantitative and qualitative differences in germline CNVs between *BRCA1*-associated and sporadic ovarian cancer patients.

TABLE 1. List of CNVs Unique to *BRCA1*-Associated Ovarian Cancer Group

Cytoband	Start	Length (Kb)	Genomic markers	CNV type	*BRCA1* ovarian cancer patients[a] (families)	Overlapping genes
1p36.33–32	1886992	1080.9	321	Amplification	1[b]	*ACTRT2, C1orf86, C1orf93, GABRD, HES5, LOC100128003, LOC100129534, LOC115110, MMEL1, MORN1, PANK4, PEX10, PLCH2, PRKCZ, RER1, SKI, TNFRSF14, FLJ42875, KIAA1751*
1p36.32	2967886	666.3	307	Amplification	2 (2)[b]	*ARHGEF16, FLJ42875, MEGF6, MIR551A, PRDM16, TPRG1L, WDR8, FLJ42875, TP73*
1p36.32	3634218	41.5	27	Amplification	1[b]	*KIAA0495, TP73, CCDC27*
1p22.2	88867379	216.4	118	Amplification	1	*PKN2*
1q12	131939195	10981.6	45	Amplification	1	*FLJ39739, LOC100130000, LOC100286793, PPIAL4G*
1q21.1	142920843	767.0	108	Amplification	1	*C1orf152, LOC728855, LOC728875, NBPF9, PDE4DIP, PPIAL4A, PPIAL4B, PPIAL4C*
1q25.3	182456954	176.5	140	Amplification	1	*C1orf21*
1q43	235562021	1044.5	874	Amplification	2 (1)	*LOC100130331, ZP4, RYR2*
3p14.1	67590604	133.2	81	Deletion	1	*SUCLG2*
4p14	39012069	167.5	76	Amplification	1	*KLB, LIAS, LOC401127, RPL9, UGDH, RFC1*
4p13	42600475	516.6	364	Amplification	1	*GRXCR1*
4q32.1	156809227	23.3	45	Deletion	1	*GUCY1A3*
6q26	162272312	128.2	113	Deletion	1	*PARK2*
7q31.32	122386377	329.1	254	Amplification	1	*SLC13A1, TAS2R16*
7q36.1	149174693	7.5	6	Amplification	1[b]	*ZNF862*
8q12.3	63510906	58.0	44	Deletion	2 (2)[b]	*NKAIN3*
8q22.2	101181884	277.9	135	Deletion	1[b]	*FBXO43, POLR2K, RNF19A, SPAG1, RGS22*
8q24.3	143194942	708.6	290	Amplification	1[b]	*ARC, BAI1, C8orf55, JRK, LY6D, LY6K, LYNX1, LYPD2, NCRNA00051, PSCA, SLURP1, TSNARE1*
9p21.2	28036146	7.0	5	Deletion	1[b]	*LINGO2*
9p13.1	38352179	417.5	390	Amplification	1	*ALDH1B1, ANKRD18A, C9orf122, IGFBPL1*
9p34.3	138083118	1539.2	421	Amplification	1	*ABCA2, AGPAT2, ANAPC2, C8G, C9orf139, C9orf140, C9orf142, C9orf163, C9orf167, C9orf169, C9orf172, C9orf173, C9orf69, C9orf75, C9orf86, CARD9, CLIC3, COBRA1, DNLZ, DPP7, EDF1, EGFL7, ENTPD2, ENTPD8, EXD3, FAM166A, FAM69B, FBXW5, FUT7, GPSM1, GRIN1, INPP5E, KIAA1984, LCN10, LCN12, LCN15, LCN6, LCN8, LCNL1, LHX3, LOC100131193, LOC100289341, LOC26102, LRRC26, MAMDC4, MAN1B1, MIR126, MRPL41, NDOR1, NELF, NOTCH1, NOXA1, NPDC1, NRARP, PHPT1, PMPCA, PNPLA7, PTGDS, QSOX2, RNF208, SDCCAG3, SEC16A, SLC34A3, SNAPC4, SNHG7, SNORA17, SNORA43, SSNA1, TMEM141, TMEM203, TRAF2, TUBB2C, UAP1L1, WDR85, ZMYND19, ARRDC1, NACC2*
10p11.23	30850068	137.7	138	Amplification	1	*LYZL2*
10q21.3	68086926	151.1	118	Deletion	1	*CTNNA3*
11p15.5	1852541	575.6	227	Amplification	1[b]	*ASCL2, C11orf21, CD81, H19, IGF2, IGF2AS, INS, INS-IGF2, LOC100133545, MIR483, MIR675, MRPL23, TH, TNNT3, TRPM5, TSPAN32, TSSC4, KCNQ1, LSP1*
15q24.2–3	86853024	953.6	327	Amplification	1[b]	*ACSF3, APRT, C16orf81, CBFA2T3, CDH15, CDT1, CTU2, CYBA, FAM38A, GALNS, IL17C, MGC23284, MVD, PABPN1L, RNF166, SNAI3, TRAPPC2L, ZC3H18, ZFPM1, ZNF469*
18q12.2	35402353	102.2	50	Deletion	1	*LOC647946*

(Continued)

TABLE 1. List of CNVs Unique to *BRCA1*-Associated Ovarian Cancer Group (Continued)

Cytoband	Start	Length (Kb)	Genomic markers	CNV type	*BRCA1* ovarian cancer patients[a] (families)	Overlapping genes
19p13.3	41910	1431.1	353	Amplification	1[b]	*ABCA7, ADAMTSL5, APC2, ARID3A, ATP5D, AZU1, BSG, C19orf20, C19orf21, C19orf22, C19orf23, C19orf24, C19orf25, C19orf26, C19orf6, C2CD4C, CDC34, CFD, CIRBP, CNN2, DAZAP1, EFNA2, ELANE, FGF22, FLJ45445, FSTL3, GAMT, GPX4, GRIN3B, GZMM, HCN2, HMHA1, KISS1R, LPPR3, MADCAM1, MED16, MIDN, MIER2, MUM1, NDUFS7, ODF3L2, OR4F17, PALM, PCSK4, POLR2E, POLRMT, PPAP2C, PRSSL1, PRTN3, PTBP1, REEP6, RNF126, RPS15, SBNO2, SHC2, STK11, THEG, WDR18*
19q13.41	58870566	149.4	108	Amplification	1[b]	*NLRP12*
19q13.43	61144359	1050.6	727	Amplification	1	*GALP, MIMT1, NLRP5, NLRP8, PEG3, PEG3AS, ZFP28, ZIM2, ZNF444, ZNF470, ZNF471, ZNF542, ZNF582, ZNF583, ZNF667, ZNF71, ZNF787, ZNF835, ZSCAN5A, ZSCAN5B*
Xp22.2–21.13	16903210	736.8	440	Amplification	4 (2)	*NHS, REPS2*
Xp21.3	28703508	109.2	100	Amplification	4 (2)	*IL1RAPL1*

[a]Numbers of *BRCA1* families were provided in the parenthesis.
[b]This means that this CNV was not detected in other member of the same family.

Our data show no significant difference in CNV frequency between *BRCA1*-associated ovarian cancer patients and healthy women (Fig. 2). Previous studies using low-resolution genetic analysis reported that allelic imbalance is increased threefold in normal breast epithelium from *BRCA1* mutation carriers, compared to controls (Larson et al., 2005; Clarke et al., 2006). Recently, Rennstam et al. (2010) used high-resolution array-based comparative genomic hybridization to show that genomic alterations in histopathologically normal breast tissue from *BRCA1* mutation carriers are more frequent than in the normal breast tissue of age-matched controls. In addition, it has been reported that different tissues vary in genomic copy numbers in the same individual (Piotrowski et al., 2008). Therefore, epithelial cells in breast or ovarian tissue might be more influenced than lymphocytes by *BRCA1* haploinsufficiency. In the future, analysis of genomic CNVs in both normal ovarian epithelium cells and lymphocytes from *BRCA1* mutation carriers who undergo prophylactic salpingo-oophorectomy will be required.

Deletions in primary lymphocyte DNAs from *BRCA1*-associated ovarian cancer patients were found more frequently than those from sporadic ovarian cancer patients or normal healthy women (Fig. 2). The use of an ultra-high-resolution SNP array allowed small-scale CNVs to be detected. The total number of small-scale deletions in the *BRCA1* group was also higher than in the other groups, as was the total number of deletions. These findings are consistent with Rennstam's result, where deletions were more frequently observed than amplifications in histopathologically normal breast tissues from *BRCA1* mutation carriers (Rennstam et al., 2010). It is thought that loss of homologous recombination by inactivation of BRCA1 results in inappropriate repair of double-strand DNA breaks via nonhomologous end-joining and single strand annealing, which leads to genomic instability through increased deletions or translocations (Moynahan et al., 1999; Venkitaraman, 2002; Turner et al., 2004; Walsh et al., 2008). Cousineau and Belmaaza (2007) indicated that breast cancer MCF7 cells with *BRCA1* haploinsufficiency display reduced efficiency of DNA double-strand break repair via homologous recombination. It is necessary to determine whether the above-described mechanisms in epithelial cells apply to other cell types such as lymphocytes. Shlien et al. (2008) reported that the CNV frequency in primary lymphocyte DNA is significantly increased in *TP53* mutation carriers compared to normal controls or the *TP53* wild-type group, likely owing to *TP53* haploinsufficiency, and proposed a model for CNV generation in tumorigenesis: an individual at risk of developing early-onset cancer such as LFS has an excess of CNVs and acquires more CNV regions in DNA from normal cells as time passes (Shilien and

Malkin, 2009). Thus, the predominance of deletions in the *BRCA1* group observed in our analysis implies that *BRCA1* haploinsufficiency might affect genome-wide CNV profiles of germline DNAs in *BRCA1*-associated ovarian cancer patients. However, we could not clarify this phenomenon in this study because blood samples for genomic DNAs were obtained only after diagnosis of ovarian cancer. In the future, longitudinal analysis of CNVs of normal DNA in the same samples should allow us to identify the presence of 'acquired CNVs' in cancer patients with *BRCA1* mutations.

Sporadic ovarian cancer patients had a higher CNV frequency than *BRCA1*-associated ovarian cancer patients or healthy controls (Fig. 2). Although we examined the relationship between age and CNV frequency in each sample, there were no significant correlations between age and CNV frequency in any group or in all samples (data not shown). When we divided the sporadic group into early-stage ($n = 9$) and advanced-stage cases ($n = 24$), no significant differences in CNV frequencies between the two subgroups were detected (Mann-Whitney test, $P = 0.86$). These data suggest that inherited CNVs, but not acquired CNVs in normal lymphocyte DNAs, might contribute largely to the higher number of CNVs in sporadic ovarian cancer patients. It is interesting to note that some sporadic-unique CNVs such as 4q13.2 and 12p13.31 are shared by two sporadic cases or more (Supporting Information Table 2). Diskin et al. (2009) reported that an inherited common CNV at 1q21.1 is associated with neuroblastoma, and that there is a previously unknown neuroblastoma breakpoint family gene within the CNV at 1q21.1 that is implicated in early tumorigenesis. To clarify the significance of the higher CNV frequency in sporadic ovarian cancer patients, it is essential to consider germline CNVs not only as rare CNVs but also as copy-number polymorphisms by performing a large-scale case-control association study.

We identified 31 *BRCA1*-unique CNV regions covering 241 overlapping genes associated with three molecular and cellular functions (Table 1 and Supporting Information Table 3). A subset of the 241 overlapping genes in *BRCA1*-unique CNVs were further involved in 'purine metabolism' and '14-3-3-mediated signaling'. Nucleotide metabolism is an important pathway related to carcinogenesis and is directly associated with DNA repair, whereas 14-3-3-mediated signaling is involved in cell cycle regulation and apoptosis. Burga et al. (2009) demonstrated that *BRCA1* haploinsufficiency leads to an increased ability of clonal growth and proliferation in primary mammary epithelial cells from *BRCA1* mutation carriers. Structural variations of genes with these molecular functions in *BRCA1*-unique CNV regions might contribute to the development of the unique biological characteristics in *BRCA1*-mutated cells. Further functional analysis is required to examine whether the overlapping genes can function as *BRCA1*-associated ovarian cancer-modifying genes.

Although several studies related to somatic CNVs of tumor DNAs previously reported that *BRCA1*-associated ovarian cancers show a higher frequency of somatic CNVs than sporadic ovarian cancers (Patael-Karasik et al., 2000; Zweemer et al., 2001; Israeli et al., 2003; Ramus et al., 2003; Walsh et al., 2008), differences in genetic CNV background between *BRCA1* and sporadic groups have received little discussion. This study shows that genomic profiles of CNV in *BRCA1* carriers are qualitatively distinct from those in sporadic ovarian cancer patients. The present finding may provide a first step to evaluate the association of germline *BRCA1* mutations with genomic CNV profiles in ovarian cancer patients.

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ORIGINAL ARTICLE *Inhibitors*

Clinical pharmacological study of a plasma-derived factor VIIa and factor X mixture (MC710) in haemophilia patients with inhibitors – Phase I trial

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Summary. MC710, a combined product of plasma-derived activated factor VII (FVIIa) and factor X (FX) at a protein weight ratio of 1:10, is a novel bypassing agent for haemostasis in haemophilia patients with inhibitors. In this study, pharmacokinetic (PK), pharmacodynamic (PD) parameters and safety of single doses of MC710 were investigated in 11 male haemophilia patients with inhibitors in a non-bleeding state. This was a multi-centre, open-labelled, non-randomized, active controlled crossover, dose-escalation study of five doses (20–120 μg kg^{-1} of FVIIa) with re-administration of different MC710 dosages to the same subjects. The active controls were NovoSeven (120 μg kg^{-1}) and/or FEIBA (50 and 75 U kg^{-1}) which were used to compare PD parameters. The area under the curve (AUC) and maximum plasma concentration (C_{max}) of MC710 active ingredients increased dose-dependently within the range of 20 and 120 μg kg^{-1}. After administration of MC710, activated partial thromboplastin time (APTT) was dose-dependently improved and prothrombin time (PT) was shortened to approximately 6 s at 10 min, and APTT improvement and PT shortening effects were maintained until 12 h after administration of MC710 at all doses. No serious or severe adverse event was observed after administration of MC710; furthermore, several diagnostic marker values and those changes did not indicate any signs of disseminated intravascular coagulation (DIC). These results suggest that MC710 would have haemostatic potential equal to or greater than NovoSeven and FEIBA and was be tolerable when given at doses up to 120 μg kg^{-1}.

Keywords: factor VIIa, factor X, haemophilia, inhibitors, bypassing agents, PK/PD

Introduction

Bleeding in haemophilia patients with inhibitors is mainly controlled by bypassing agents, recombinant activated factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsværd, Denmark) and activated prothrombin complex concentrates (APCC; FEIBA®, Baxter International Inc., Deerfield, IL, USA), however; those agents cannot always provide complete haemostasis. Currently, an improved regimen including combination therapy of rFVIIa and APCC are proceeding on the development [1–3]. On the other hand, a rFVIIa analogue in which several amino acids are mutated has been developed to strengthen the haemostatic effect

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