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Mutational Analysis of *BRCA1* Gene in Ovarian and Breast-ovarian Cancer Families in Japan

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We analyzed the alteration of *BRCA1* in DNA obtained from 83 individuals of 13 Japanese site-specific ovarian cancer families and 6 breast-ovarian cancer families. Six germline mutations were detected in 7 families, which consisted of 4 breast-ovarian cancer and 3 site-specific ovarian cancer families, by single-strand conformation polymorphism analysis, followed by direct sequence determination. The mutations included three frameshifts, two nonsense mutations, and one missense mutation causing loss of a zinc-binding motif. The frequency of loss of heterozygosity at the microsatellite markers on the *BRCA1* gene was 57% (8 of 14 cases) in site-specific ovarian cancer families, and 100% (6 of 6 cases) in breast-ovarian cancer families. All tumors of the patients carrying a mutation of *BRCA1* showed deletion of wild-type alleles, implicating *BRCA1* as a tumor suppressor gene. These results suggest that germline mutations of the *BRCA1* gene play an important role in the carcinogenesis of breast and/or ovarian cancer in a majority of breast-ovarian cancer families and in some site-specific ovarian cancer families.

Key words: *BRCA1* — Mutation — Ovarian cancer — Breast-ovarian cancer — Japanese

Ovarian cancer is the most lethal gynecological malignancy in Japan. Although chemotherapy using a cisplatin-containing regimen is reported to be relatively effective in the treatment of epithelial ovarian cancer, the median survival rate for epithelial ovarian cancer patients with stage III or stage IV is about 2 years.[1)] About two-thirds of the patients are in an advanced stage at the time of diagnosis, and this is a major reason for the poor prognosis. Some method for early detection of ovarian cancer is needed to improve the prognosis.

The gene *BRCA1*, which is thought to be related to susceptibility to breast and ovarian cancer, was localized on chromosome 17q by genetic linkage analysis,[2)] and identified by positional cloning methods.[3)] Mutation of *BRCA1* is thought to account for approximately 45% of breast cancer families and about 80% of families with both early-onset breast cancer and ovarian cancer.[4)] It was reported that familial site-specific ovarian cancer in a western country was linked to *BRCA1*.[5)] In DNA of sporadic ovarian tumors, a low incidence of somatic mutation was reported, though loss of heterozygosity (LOH) had been frequently detected.[6, 7)]

Many different mutations of *BRCA1* have been reported in approximately half of investigated families with breast-ovarian cancers. Some of the mutations of *BRCA1* showed geographical spread.[8–10)]

In Japan, 11 Japanese breast cancer families showed negative linkage to *BRCA1*,[11)] while germline mutations were detected in only two of 20 Japanese breast and breast-ovarian cancer families.[12)] The analysis of 1000 Japanese breast cancer patients revealed only 8 cases of germline mutations.[13, 14)] In addition, in 4 of 76 Japanese ovarian cancers, germline mutations were detected.[15)] These data suggest that the genetic alteration in hereditary breast-ovarian cancer in Japan may differ from that in western countries previously studied.

We analyzed the alteration of *BRCA1* in 13 Japanese site-specific ovarian cancer families and 6 breast-ovarian cancer families to elucidate the role of *BRCA1* in carcinogenesis in these families.

MATERIALS AND METHODS

Families We examined 13 site-specific ovarian cancer families and 6 breast-ovarian cancer families. The criteria for a site-specific ovarian cancer family were: two or

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more members affected with ovarian cancer in the first-degree relatives and no breast cancer case in the third-degree relatives. When the family had at least one breast cancer case in the third-degree relatives, it was classified as a breast-ovarian cancer family.

DNA extraction After having obtained informed consent, we took whole blood samples and extracted genomic DNA by using the phenol/chloroform method.[16] For detection of LOH, DNA of the tumor was extracted from a paraffin-embedded block. The extraction of DNA from tumors was carried out in the same way.

Mutational analysis by single strand conformation polymorphism (SSCP) analysis DNAs from all ovarian cancer patients, breast cancer patients and a healthy woman of each family were analyzed by SSCP in 21 exons (not exons 1, 4, and 11). Polymerase chain reaction (PCR) was performed using a pair of primers, both end-labeled with FITC or Cy5 (Pharmacia Biotech in Japan, Tokyo). PCR products were amplified from 50 ng of genomic DNA and electrophoresed through polyacrylamide gel (5% acrylamide, 0.1% methylenebisacrylamide, 5–10% glycerol), which was kept at 15°C. PCR products were analyzed on an autosequencer using Fragment Manager software (Pharmacia Biotech in Japan).

Mutational analysis by direct sequencing When we observed shifted bands in SSCP analysis, we performed direct sequencing of them. Exon 11 of all affected members was analyzed by direct sequencing.

PCR was performed in four overlapping fragments of exon 11 from 100 ng of genomic DNA. Other exons were amplified with an end-labeled (FITC or Cy5) primer.[17] The PCR products were sequenced by the dideoxy method using the Autocycle sequencing kit (Pharmacia Biotech in Japan). PCR products were electrophoresed in 6% polyacrylamide gel and analyzed with an automatic sequencer (ALF Sequencer II, ALF Express).

Analysis of LOH in tumor DNA For the detection of LOH, we used three microsatellite markers, D17S855, D17S1322 and D17S1325, located on the *BRCA1* gene.[3, 18] One of a pair of PCR primers was end-labeled with FITC. PCR amplification using 50 ng of normal and tumor DNA was carried out, and the products were mixed with 95% formamide, denatured, subjected to electrophoresis in 6% polyacrylamide gels and analyzed on an autosequencer using Fragment Manager software. A decrease of more than half in the band intensity in a tumor sample compared with that of genomic DNA was determined as LOH.

RESULTS

Characterizations of families The characteristics of the families are shown in Table I. All of the site-specific ovarian cancer families had two patients (total 26 patients), and the average age at diagnosis was 48.5 years (range; 24–79 years). Breast-ovarian cancer families had more than two patients with ovarian cancer (total 17 patients) and at least one patient with breast cancer (total 10 patients with breast cancer) including 4 early-onset/under 45 years old and 6 late-onset/more than 45 years old cases. The average age at diagnosis of ovarian cancer in 6 breast-ovarian cancer families was 49.5 years (range; 38–83 years) and that of breast cancer was 50.2 years (range; 27–67 years). One patient in Family 022 was affected with breast and ovarian cancer. There was no case of male breast cancer and no case of bilateral breast cancer. The pathological types of classifications of ovarian cancer based on FIGO (1988) were as follows; serous (37/43), mucinous (2/43), endometrioid (2/43), and clear cell (2/43). The clinical stage of patients at diagnosis were as follows; stage I (7/43), stage II (1/43), stage III (21/43), stage IV (4/43), and unknown (10/43).

Detailed pathological reports were available for 5 of 10 patients with breast cancer. All were invasive ductal carcinoma. The clinical stages were as follows; stage II (4 cases), and stage III (1 case).

Family 002 had two patients with ovarian cancer, but DNA of an affected member was unavailable.

Mutations of *BRCA1* We screened the whole coding region except exons 1, 4 and 11 by SSCP, and detected a total of 62 shifted bands in all analyzed exons (data not shown). Direct sequencing was performed in these cases.

Six independent germline mutations were detected in 7 families (Table II). Five of them were predicted to result in protein truncation; three frameshifts and two nonsense mutations. Another mutation of one base substitution, changing cysteine to glycine in exon 3, was expected to result in loss of a zinc-binding motif. The pedigrees of the seven families with mutations of *BRCA1* are shown in Fig. 1.

Deletion of A at nucleotide 241 in exon 3 was detected in two affected members of Family 001. This is a newly detected frameshift, which is expected to lead to protein truncation.

A missense mutation (T to G at nucleotide 300 in exon 5) was observed in three affected members of Family 010, but not in a healthy individual.

A nonsense mutation (T to A at nucleotide 307 in exon 5) was detected in Families 015 and 020. This substitution is considered to change leucine at codon 63 to a stop codon (ochre). Two affected sisters in Family 015 had this mutation. However, in one patient with ovarian cancer (No. 081) in Family 020, a missense mutation (A to G substitution at nucleotide 4220 in exon 12) was observed. This substitution was considered to change Glu to Asp and was not detected in any other member of the pedigree (No. 082, 083 and 085) tested.

Table I. Characterization of Japanese Ovarian and Breast-ovarian Cancer Families

Family	No. of ovarian cancer patients	Age at diagnosis of ovarian cancer patients (yr)	No. of breast cancer patients	Age at diagnosis of breast cancer patient(s) (yr)	No. of patients examined for LOH
Site-specific ovarian cancer families					
001	2	57, 79	—	—	2
002	2	42, 61	—	—	0
003	2	46, 78	—	—	1
004	2	30, 42	—	—	1
005	2	40, 59	—	—	1
006	2	40, 47	—	—	2
011	2	41, 44	—	—	2
014	2	49, 55	—	—	1
015	2	50, 55	—	—	2
016	2	40, 42	—	—	1
017	2	55, 65	—	—	1
019	2	24, 47	—	—	0
024	2	24, 50	—	—	0
Subtotal	26	48.5			14
Breast-ovarian cancer families					
010	3	53, 53, 63	2	30, 65	0
012	4	39, 47, 47, 55	1	52	2
018	2	40, 46	1	51	0
020	3	38, 41, 51	3	27, 43, 74	0
021	3	38, 44, 50	1	67	3
022	2	54, 83[a)]	2	35, 58[a)]	1
Subtotal	17	49.5	10	50.2	6
Total	43		10		20

a) The patient was affected with both ovarian and breast cancer.

Table II. Germline Mutations in Japanese Site-specific Ovarian and Breast-ovarian Cancer Families

Family	Exon	Nucleotide	Mutation	Amino acid change	Predicted effect
001	3	241	deletion of A	codon stop	protein truncation
010	5	300	T to G	Cys to Gly	lose zinc-binding motif
012	11	3759	G to T	Glu to amber	protein truncation
014	11	2073	deletion of A	codon stop	protein truncation
015	5	307	T to A	Leu to ochre	protein truncation
020	5	307	T to A	Leu to ochre	protein truncation
021	12	4239	deletion of AG	codon stop	protein truncation

Kindreds in Family 012 had a nonsense mutation which changes G to C at nucleotide position 3759 in exon 11. This substitution changes Glu to amber at codon 1214 (Glu 1214 stop), and was observed in 3 affected members with ovarian cancer and a patient with breast cancer, but not in a healthy woman.

Deletion of A at nucleotide 2073, resulting in the stop at codon 700, was observed in two affected members of Family 014, but not in a healthy individual.

A frameshift (deletion of AG at nucleotide 4239 in exon 12) was detected in two affected members in Family 021. DNA of patient No. 293 was unavailable and mutational analysis of patient No. 293 was not performed.

LOH analysis DNAs were successfully extracted from only 20 tumor specimens from 20 patients among 43 patients because of an insufficient amount of tumor specimen or difficulty of DNA extraction. Analysis of LOH with the markers located on the *BRCA1* gene was performed on all available tumors; 14 tumors from 10 site-specific ovarian cancer families and 6 tumors from 3 breast-ovarian cancer families (Table III). LOH at the marker D17S855 was detected in 80% (8 of 10 informa-

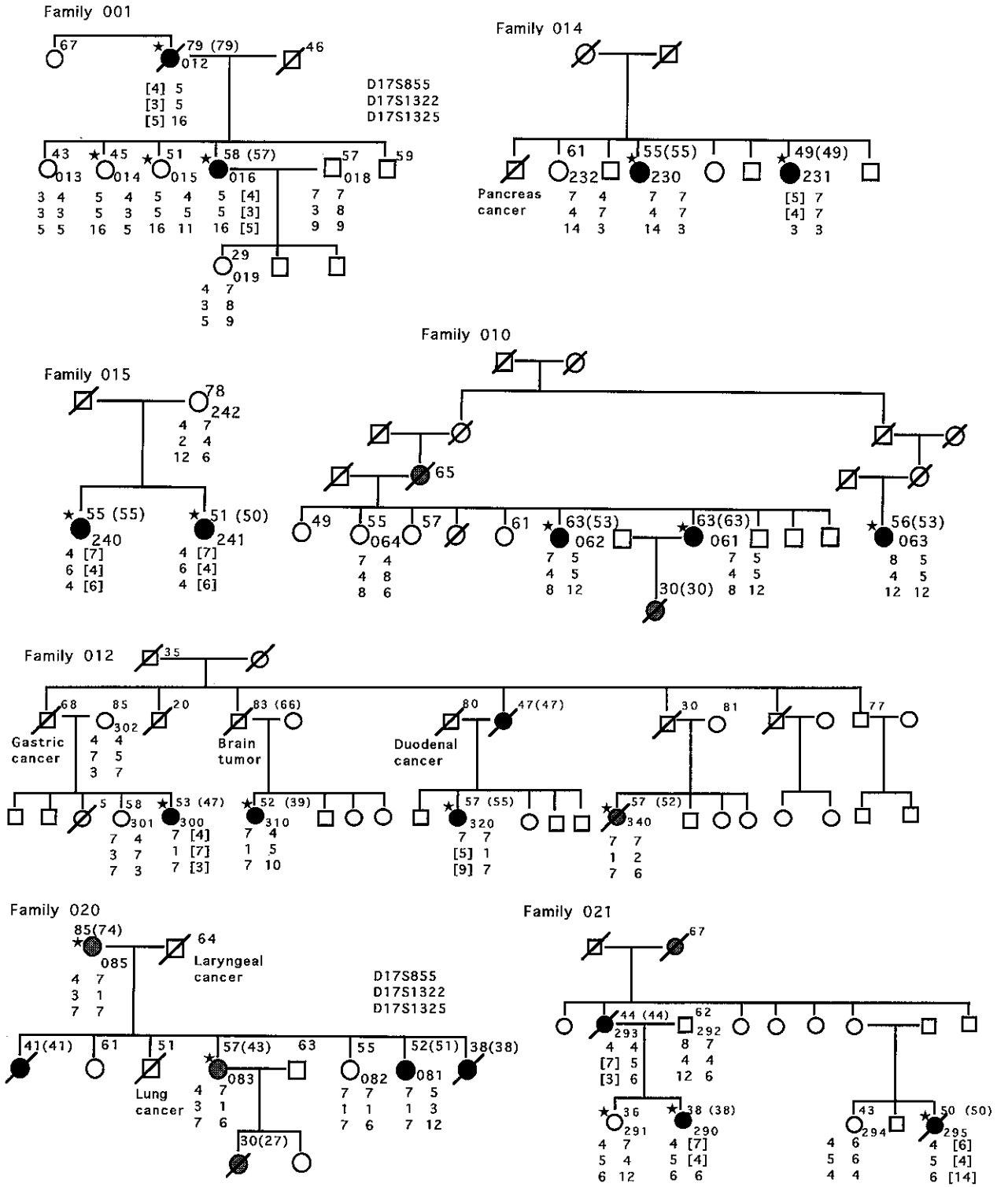

Fig. 1. Pedigrees of seven families with mutation of *BRCA1* are shown. Haplotypes of microsatellite markers located on *BRCA1* are indicated below each individual. ○, female; □, male; ●, ovarian cancer; ⊗, breast cancer; ★, member with mutation of *BRCA1*. Age is indicated by the number to the upper right of each symbol. A number in brackets ( ) indicates age at diagnosis. Haplotypes shown in this figure are, from top to bottom, D17S855, D17S1322 and D17S1325. A number in square brackets [ ] indicates a deleted allele.

Table III. LOH Data of 20 Tumors from Japanese Ovarian and Breast-ovarian Cancer Families

	Patient No.	Family	D17S855	D17S1322	D17S1325
Site-specific ovarian cancer	012	001	●	●	●
	016	001	●	●	●
	008	003	△	△	△
	091	004	○	○	○
	052	005	UI	○	○
	071	006	UI	○	○
	072	006	UI	○	○
	210	011	△	△	△
	211	011	△	△	△
	231	014	●	●	UI
	240	015	●	●	●
	241	015	●	●	●
	250	016	○	○	○
	261	017	UI	○	○
Breast-ovarian cancer	300	012	●	●	●
	320	012	UI	●	●
	290	021	●	●	UI
	293	021	●	UI	●
	295	021	●	●	●
	351	022	△	△	△

●, wild-type allele was deleted; △, LOH was detected; ○, retention of heterozygosity; UI, uninformative.

tive cases) of patients in site-specific ovarian cancer families, and 100% (5 of 5 informative cases) of patients in breast-ovarian cancer families. Furthermore, allele losses at the markers D17S1322 and D17S1325 were detected in 57% and 54% of site-specific ovarian cancer, and 100% and 100% of breast-ovarian cancer families, respectively. The frequencies of allelic losses detected on the *BRCA1* gene (at least one of the markers D17S855, D17S1322 and D17S1325) was 57% (8 of 14 cases) in site-specific ovarian cancer families and 100% (6 of 6 cases) in breast-ovarian cancer families.

LOH was detected in 5 of 5 families with mutation of *BRCA1*. Typing of DNA polymorphisms in these pedigrees was analyzed with the same microsatellite markers, and it appeared that the deleted alleles were wild-type alleles (Fig. 1). In members of Family 003, Family 011, and Family 022, LOH was detected in these markers located on *BRCA1*, but no mutation of *BRCA1* was found.

DISCUSSION

We examined 19 families containing two or more members with ovarian cancer to analyze the influence of the *BRCA1* gene on carcinogenesis of ovarian and breast cancer.

SSCP and direct sequence analysis revealed 6 germline mutations in 7 families. The substitution of T to A at nucleotide 307 (Leu 63 ochre) observed in Families 015 and 020 had previously been reported in 2 site-specific breast cancer families, and a nonsense mutation at nucleotide 3759 (Glu 1214 amber) was found in a Japanese sporadic breast cancer case.[13, 14] Two other mutations have been reported in western countries; deletion of A at nucleotide 2073 (codon 654) which results in a stop at the following codon (codon 700)[19] and a missense mutation (T to G at nucleotide 300 in exon 5)[17, 20–24] which leads to loss of a zinc-binding motif. We detected two novel mutations in the present study; a deletion of A at nucleotide 241 (241 del A) in Family 001 and a deletion of AG at nucleotide 4239 (4239 del AG) in Family 021.

Various *BRCA1* mutations have been identified,[25] though only a few have been detected repeatedly. One such mutation, a two-base-pair deletion at position 185 in exon 2 of *BRCA1* (185 del AG) identified in more than 20 Jewish families with familial breast and/or ovarian cancer, is observed in about one percent of Ashkenazi Jews.[9, 10] These studies suggest that some mutations of *BRCA1* are segregated geographically and racially. In the Japanese population, 14 germline mutations of *BRCA1* were previously found in 2 site-specific breast cancer families, 4 sporadic ovarian cancer patients and 8 sporadic breast cancer patients.[12–15] Based on these results and our findings, no germline mutation unique to the Japanese has yet been found. However, interestingly, the substitution of T to A at nucleotide 307 (Leu 63 ochre)

was detected in 4 families; two site-specific breast cancer families and two breast-ovarian cancer families. These families were unrelated, and it is unclear whether the carriers of these mutations have common ancestors.

In this experiment, we analyzed 6 families with breast-ovarian cancer. Four of them carried a mutation of *BRCA1* in breast and ovarian cancer and LOH of the wild-type allele was noted in all cases tested. These findings clearly suggest the involvement of *BRCA1*. In two families we could not detect mutation of the coding regions of *BRCA1* (Families 018 and 022), but LOH was observed at these markers, suggesting that *BRCA1* might play a role in carcinogenesis of ovarian cancer in these families. Several explanations might be possible for the results obtained from the latter two families, such as intronic mutation causing splicing aberration, lower sensitivity of SSCP, and LOH independent of *BRCA1* mutation. Substitution at nucleotide 4220 was detected only in one affected member with ovarian cancer in Family 020 (No. 081), but not in other members (No. 082, 083 and 085) in this pedigree. This substitution may not be due to polymorphism of the *BRCA1* gene, as it was not detected in DNA from 30 healthy Japanese volunteers. However, its significance remains unclear at present.

As for site-specific ovarian cancer, among 13 families with at least two ovarian cancer patients, mutations of *BRCA1* were found in only 3 families.

Recently, the *BRCA2* gene on chromosome 13 was cloned, and it was reported to be involved in 40–45% of site-specific breast cancer families,[26] but not to confer susceptibility to ovarian cancer.[27] We have not examined the involvement of *BRCA2* in our cases.

Our results suggest that breast or ovarian cancer in breast-ovarian cancer families can be attributed to germline mutation and LOH of the *BRCA1* gene, and such changes also play an important role in at least a part of ovarian cancer in site-specific ovarian cancer families. Further genetic analysis is needed to clarify the mechanism of carcinogenesis in ovarian cancer families in whom we could not detect specific mutation. However, the basic difficulty with this study is insufficient data, because of the small number of affected cases in a family. In such families, persons without inherited susceptibility to disease may be coincidentally affected, and this might obscure the genetic linkage. Other approaches such as lmlink (the combination of linkage and LOH analysis)[28,29] and non-parametric sibling-pair linkage analysis[30] may be useful for further investigation in these families.

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