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ORIGINAL ARTICLE

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High proportion of missense mutations of the *BRCA1* and *BRCA2* genes in Japanese breast cancer families

Received: September 4, 1997 / Accepted: October 28, 1997

Abstract Mutations in either of two recently identified genes, *BRCA1* and *BRCA2*, are thought to be responsible for approximately two-thirds of all cases of autosomal-dominantly inherited breast cancer. To examine the nature and frequency of *BRCA1* and *BRCA2* mutations in Japanese families exhibiting a high incidence of breast cancer, we screened 78 unrelated families in this category for mutations of these two genes. Examining the entire coding sequences as well as exon–intron boundaries of both genes by polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP) and multiplex-SSCP analysis, we identified possible disease-causing alterations in *BRCA1* among affected members of 15 families and in *BRCA2* in another 14 families. In 15 of those 29 families, the affected individuals carried missense mutations, although most germline mutations reported worldwide have been deletions or nonsense mutations. Our results, indicating that missense mutations of *BRCA1* and *BRCA2* tend to predominate over frameshifts or nonsense mutations in Japanese breast cancer families, will contribute significantly to an understanding of mammary tumorigenesis in Japan, and will be of vital importance for future genetic testing.

Key words *BRCA1* · *BRCA2* · Breast cancer family · Germline mutation · Missense mutation

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Introduction

Breast cancer is one of the most common malignancies among women, and its cumulative risk by age 85 is 1 in 8 women in the United States and 1 in 40 women in Japan (Newman et al. 1988; Lynch 1990; American Cancer Society 1994). Epidemiological studies suggest that inherited susceptibility may account for 5%–10% of all breast cancers, and that approximately one in 200 American women may carry a predisposing allele. Recently, two genes responsible for a major proportion of hereditary breast cancers, *BRCA1* on chromosome 17q21 (Miki et al. 1994) and *BRCA2* on chromosome 13q12–13 (Wooster et al. 1995), were identified, and between them, these two genes are thought to cause approximately two-thirds of all breast cancers among families in the United States and Europe that show a pattern of autosomal-dominant transmission of this disease. Among women carrying constitutional *BRCA1* mutations, the lifetime risk of breast cancer exceeds 80%, and the risk of ovarian cancer approaches 50% (Ford et al. 1994). Similarly, the risk for breast cancer in carriers of *BRCA2* mutations is very high; moreover, several other cancers appear to be a part of the *BRCA2* spectrum, including male breast cancer (Couch et al. 1996; Thorlacius et al. 1996), pancreatic cancer (Teng et al. 1996; Goggins et al. 1996), and hepatocellular carcinoma (Katagiri et al. 1996a). The risk of ovarian cancer is, however, much lower than in carriers of *BRCA1* mutations.

The incidence of breast cancer in Japan is much lower than that in the United States or western Europe, and disease onset in predisposed Japanese patients tends to occur much later in life than is the case among Caucasians (Katagiri et al. 1996b). Certain epidemiological data have implied notable differences in the background for breast carcinogenesis with respect to the Japanese population versus American or European populations (de Waard 1981; Kelsey and Berkowitz 1988). Furthermore, recurrent *BRCA1* and *BRCA2* mutations in defined subgroups (for example, persons of Ashkenazi Jewish descent) have been reported (Struewing et al. 1995; Tonin et al. 1995; Fitzgerald et al. 1996; Berman et al. 1996; Neuhausen et al. 1996; Offit et al. 1996). As the purpose of the present study was to examine the nature and frequency of *BRCA1* and *BRCA2* mutations in Japanese "breast cancer families," we screened DNA from patients in 78 unrelated families by polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP) and multiplex-SSCP analysis.

Materials and methods

Subjects

The families comprising the population sample for the present study were identified in collaboration with the Japanese Breast Cancer Society and the Japanese Familial Tumor Society. Seventy-eight unrelated families showing a pattern of inheritance of breast cancer were evaluated. For each family, a pedigree was prepared on the basis of a family member known to be affected.

Occurrence of cancer within each pedigree was confirmed by obtaining medical records and pathology reports for all available family members whether living or deceased. The criteria for selecting "breast cancer families" for this study were as follows: (a) at least three first-degree family members with breast cancer or (b) two or more first-degree family members with breast cancer, either early-onset, bilateral, or accompanied by a history of primary cancer(s) of other organs. Blood samples were obtained from affected family members through the aforementioned Societies. Genomic DNAs were extracted from fresh blood under standard protocols (Kunkel et al. 1977).

Mutation screening

SSCP-analysis. The entire coding sequences of *BRCA1* and *BRCA2*, and associated exon–intron boundary sequences, were examined by PCR-SSCP analysis. The PCR primers used for PCR-SSCP analysis have been described elsewhere (Katagiri et al. 1996b, Miki et al. 1996).

Each genomic DNA (50 ng) was amplified in a reaction mixture containing 10 ml of 1 × PCR buffer (25 mM TAPS, 50 mM KCl, 2 mM $MgCl_2$, and 1 mM β-mercaptoethanol), 20 mM dNTPs, 5 pmol primers, 2 mCi of α[^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml), and 0.5 units of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany). PCR conditions were 1 cycle at 94°C for 2 min, then 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with final elongation at 72°C for 5 min. Each reaction mixture was diluted with 50 ml of 95% formamide dye and 20 mM ethylenediaminetetraacetic acid (EDTA), incubated at 85°C for 5 min, and electrophoresed in a 6% polyacrylamide gel containing 5% glycerol and 0.5 × 90 mM Trisborate/2 mM EDTA (TBE) maintained at 16°C. The gel was dried and autoradiographed with an intensifying screen. When variant bands were detected in SSCP analysis, PCR products from families were subcloned into pT7-Blue T (Novagen, Madison, WI, USA). At least 100 clones were pooled together and DNAs were extracted as sequence templates. Their nucleotide sequences were determined by dideoxy-chain termination with T7 DNA polymerase, using gene-specific primers to identify the nature of the mutation. All results were confirmed by two independent experiments.

PCR multiplex-SSCP analysis. A multiplex-SSCP analysis was performed with exons 10, 11, 14, 18, and 27 of the *BRCA2* gene. The PCR was carried out under the same conditions as before except that extension was performed for 2 min. Each amplified fragment was digested by a combination of restriction enzymes: *Eco*RI, *Dra*I for exon 10; *Rsa*I, *Dra*I for exon 11-A; *Dpn*I, *Fok*I for exon 11-B; *Dpn*I, *Ssp*I, *Sau*96I for exon 11-C; *Dra*I for exon 14; *Sau*3AI for exon 18; and *Dra*I, *Msp*I, *Sca*I, *Bcl*I for exon 27. The digested PCR products were electrophoresed under the same

conditions as before. Any SSCP variants detected in the multiplex-SSCP experiments were mapped to smaller segments (less than 400 bp in size) within the amplified region by conventional SSCP analysis. A PCR product that demonstrated an SSCP variation in the regional mapping was subcloned into pT7-Blue T and sequenced by the protocol described for the PCR-SSCP analyses. All results were confirmed by two independent experiments.

Results

Representative autoradiograms of PCR-SSCP and sequence analyses of the *BRCA1* and *BRCA2* genes are shown in Fig. 1. The DNA sequence analysis of the PCR product from Family #52 revealed a C to T transition at the second nucleotide of codon 1025 in *BRCA1*, which would result in the substitution of threonine for isoleucine (Fig. 1a). A deletion of four nucleotides, ACAG, had occurred at codons 251 and 252 in *BRCA2* in Family #18, causing a frameshift that would result in premature termination of the gene product (Fig. 1b).

By screening the entire coding regions of the *BRCA1* and *BRCA2* genes in 78 unrelated Japanese breast cancer families, we identified possible disease-associated alterations in a total of 29 (37.2%) cases: 13 different alterations in the *BRCA1* gene were seen in 15 of these families (19.2%) and 13 different alterations in the *BRCA2* gene in the other 14 (17.9%) (Table 1).

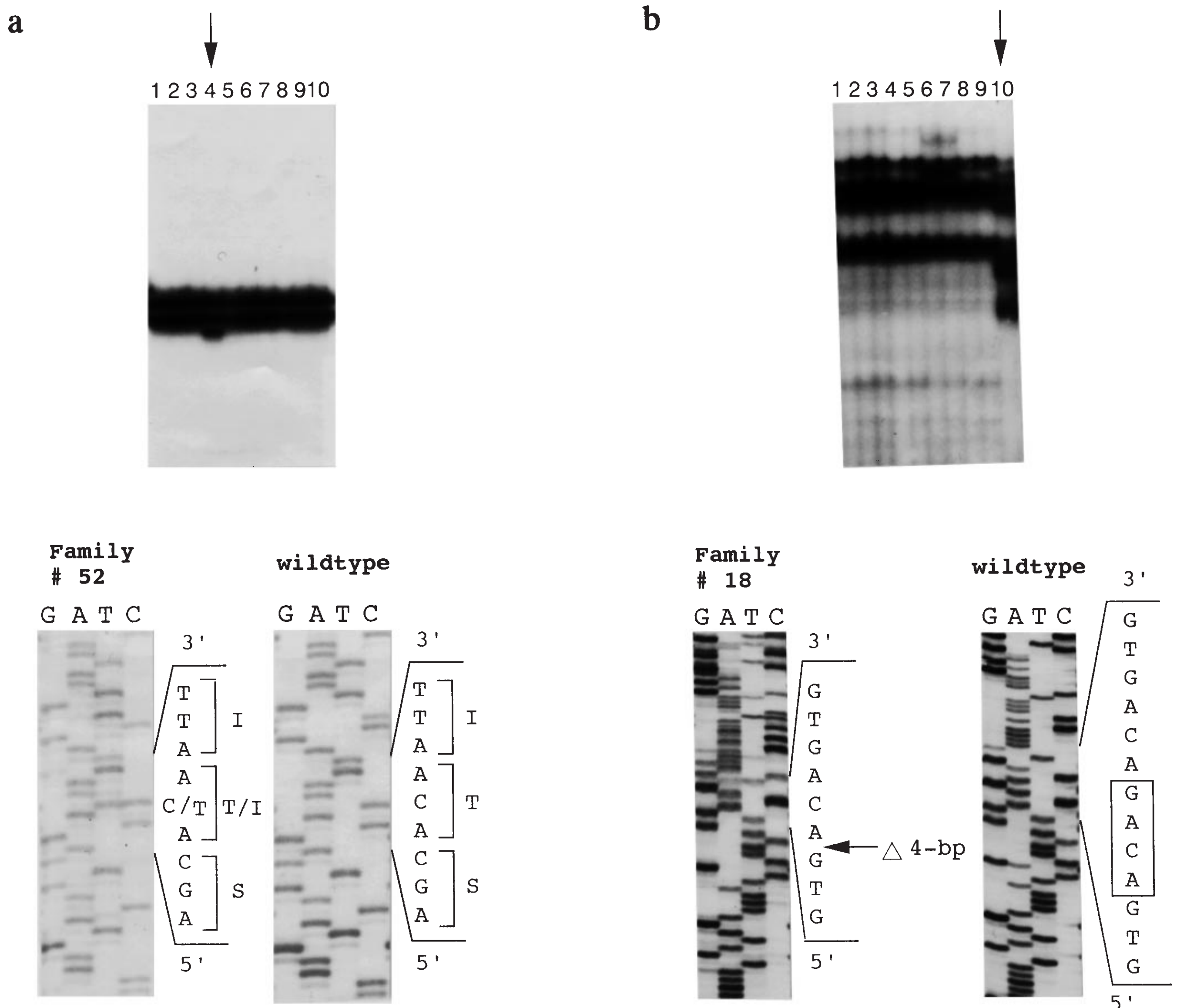

Fig. 1a,b Representative examples of polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) and sequence analysis of the *BRCA1* and *BRCA2* genes. **a** amplification of exon 11 of *BRCA1* from Family #52. **b** amplification of exon 9 of *BRCA2* from Family #18. *Upper panels*, autoradiograms of PCR-SSCP analyses. *Lower panels*, autoradiograms of sequence analyses. *Arrows* indicate sites of mutations; **a** Lane 4, **b** Lane 10

Table 1 The frequency of *BRCA1* and *BRCA2* mutations in Japanese breast cancer families

	Missense mutation	Nonsense mutation or frameshift	Intronic mutation	Total (%)
BRCA1	8	2	5	15/78 (19.2)
BRCA2	7	4	3	14/78 (17.9)
Total				29/78 (37.2)

The *BRCA1* alterations consisted of eight missense mutations, one frameshift due to 1-bp deletion (nt 1995 del), one nonsense mutation (Leu22ter), and five changes in intronic sequences close to intron–exon boundaries (Table 2).

As shown in Table 3, the *BRCA2* alterations included seven missense mutations, three frameshifts due to deletions, one nonsense mutation, and three changes in intronic sequences near splice sites. A 4-bp deletion at codons 251 and 252, causing a frameshift that would result in early termination, was found in two families (#18 and #50).

The frequency of missense mutations in both genes was much higher than other reports have indicated. As none of the missense alterations identified in the present study was present in the other 77 breast cancer families examined, in 100 sporadic breast cancer patients (Miki et al. 1996), or in familial cases reported previously, we assume that our results are likely to reflect novel disease-causing mutations. Although segregation analyses or functional analysis of the mutated products should be done to confirm whether the patients carrying these mutant alleles were in fact predisposed to breast or ovarian cancer, DNA samples from clinically affected family members unfortunately were not available to us. Moreover, since no mRNA from the patients carrying intronic mutation was available, we were unable to examine whether these mutations in introns affected splicing of the transcript. However, many reports of intronic mutations causing aberrant splicing have been presented.

The average age of onset for breast and ovarian cancer in our panel of patients with constitutional *BRCA1* mutations was 46.2 years; in patients with *BRCA2* mutations it was 49.7 (Table 4). It is apparent that breast cancers in Japanese families with *BRCA1* or *BRCA2* mutations tend to occur later than in Caucasian carriers of mutant alleles: the average age of onset for breast or ovarian cancer in patients carrying missense mutations was 47.0 and in patients carrying nonsense or frameshift mutations, 50.9; the type of mutation did not appear to influence the age of onset in the Japanese families examined. Hence, the difference in onset age among different populations is likely to reflect differences in hormonal or nutritional conditions. Many of the families we studied had members who had developed primary cancers of other organs including stomach, uterus, esophagus, colon, brain, pancreas, or bone (Tables 2 and 3).

Discussion

More than 100 different *BRCA1* mutations have been reported worldwide; data concerning *BRCA1* and *BRCA2* mutations were available to us through the breast cancer mutation database of the Breast Cancer Information Core (at http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). Among both recurrent and unique mutations of *BRCA1* described in the database, the majority are deletions or insertions of short nucleotides resulting in presumed truncation of the protein product. Although

Table 2 *BRCA1* mutations in Japanese breast cancer families

Family	Exon[a]	Codon	Nucleotide change	Coding effect[b]	Mutation	Age at onset	
						Breast or ovarian cancers	Others[c]
21	2	22	TTA – TCA	M	Leu 22 Ser	br (45), (br (39), ov (40))	ga (u)
39	2	22	TTA – TGA	N	Leu 22 Ser	br (43), br (u), br (u)	2 ut (u)
29	9−62		1-bp deletion	ND	Intronic mutation	br (45), br (39, 58)	
33	9−2		g – a	ND	Intronic mutation	br (28), (br (u), re (u))	
73	9−62		1-bp deletion	ND	Intronic mutation	br (19), br (46), br (51)	
77	9−62		1-bp deletion	ND	Intronic mutation	br (55), br (70), br (35, 45)	2 es (u)
38	10+37		1-bp insertion	ND	Intronic mutation	br (44), br (45)	
45	11	461	TTT – CTT	M	Phe 461 Leu	br (33), br (u)	2 ga (u)
78	11	465	TAT – GAT	M	Tyr 465 Asp	br (29), br (u)	
14	11	552	GGT – GTT	M	Gly 552 Val	br (48, 55), br (u), br (u)	co (u)
85	11	654	1-bp deletion	F	nt 1955 del 1	br (43), br (51), br (39, 55)	
66	11	892	TTA – TCA	M	Leu 892 Ser	br (56), br (61), br (51, 51)	ut (44)
20	11	960	GGC – GAC	M	Gly 960 Asp	br (32), br (38), br (53), br (55)	
52	11	1025	ACA – ATA	M	Thr 1025 Ile	br (48), br (49)	bra (u)
19	11	1047	GTA – GCA	M	Val 1047 Ala	br (49), br (u, u)	

[a] The positions of intronic mutations; (–) indicates upstream of the exon, (+) indicates downstream of the exon.
[b] M, missense mutation; N, nonsense mutation; F, frameshift mutation; ND, not determined.
[c] br, breast; ov, ovarian; ga, gastric; ut, uterus; re, renal; es, esophageal; co, colon; bra, brain; pa, pancreatic; os, osteosarcoma; u, unknown.
( ) indicates age at onset of cancer.

Table 3 *BRCA2* mutations in Japanese breast cancer families

Family	Exon[a]	Codon	Nucleotide change	Coding effect[b]	Mutation	Age at onset	
						Breast or ovarian cancers	Others[b]
12	3	32	TTT – CTT	M	Phe 32 Leu	br (45), male br (u, u)	
68	3	53	AAA – AGA	M	Lys 53 Arg	br (35), br (46), br (59)	pa (u)
32	3	81	TTC – CTC	M	Phe 81 Leu	br (35), br (44), br (58)	
23	7	201	CCA – CGA	M	Pro 201 Arg	br (41), br (70)	
70	8	211	GTC – GCC	M	Val 211 Ala	br (42), br (46), br (48), br (56), br (57)	
98	8	222	CCT – TCT	M	Pro 222 Ser	br (38), br (53), br (58)	
18	9	251–252	4-bp deletion	F	nt 751 del 4	br (33), br (40), br (49, 49) br (44, 58)	2 ga (u)
50	9	251–252	4-bp deletion	F	nt 751 del 4	br (43), br (47)	es (77)
13	15+5		ccc – tct	ND	Intronic mutation	br (35), br (41), ov (59)	
59	16	2534–2539	16-bp-deletion	F	nt 7602 del 16	br (45), br (59), br (67), br (82)	
25	21	2893	CAG – TAG	N	Gln 2893 ter	br (45), br (52), br (73)	
57	22+5		g – t	ND	Intronic mutation	br (37), br (39)	
97	25	3118	ATG – ACG	M	Met 3118 Thr	br (45), (br (32), os (16))	
36	26–28		a – g	ND	Intronic mutation	br (41), br (70)	

[a] The positions of intronic mutations; (−) indicates upstream of the exon, (+) indicates downstream of the exon.
[b] ( ) indicates age at onset of cancer.

Table 4 Average of onset age for breast and ovarian cancer in patients with *BRCA1* and *BRCA2* mutations

	Missense mutation (yr)	Nonsense or frameshift mutation (yr)	Total (yr)
BRCA1	46.6	46.2	46.5
BRCA2	47.8	52.4	49.7
Total	47.2	50.8	48.5

fewer *BRCA2* mutations have been reported to date, a similar tendency is emerging (Neuhausen et al. 1996; Tavtigian et al. 1996; Thorlacious et al. 1996; Phelan et al. 1996; Couch et al. 1996; Johannesdottir et al. 1996). In the present study we evaluated the nature and frequency of *BRCA1* and *BRCA2* mutations among 78 unrelated Japanese breast cancer families, and detected possible disease-causing mutations in one or the other of these genes in about 40% of the families examined. The frequency of detectable mutations in our test panel was lower than that reported in American or European studies, possibly because (a) genes other than *BRCA1* or *BRCA2* may contribute to predisposition to breast cancer in the Japanese population; (b) the promoter regions of the *BRCA1* and *BRCA2* genes, which we did not examine, may contain mutations in the other 60% of our panel; and/or (c) our criteria for "hereditary breast cancer family" were not strict enough and therefore some cases examined might have been sporadic.

Eight cases with missense mutations and five with changes in intronic sequences of the *BRCA1* gene were found. Similarly, we detected seven cases with missense mutations and three with changes in intronic sequences of *BRCA2*. As none of these alterations was present in a large number of cases reported previously, they are likely to represent novel disease-associated mutations. However, because we did not have access to RNA samples, we cannot exclude the possibility that the nucleotide changes we found in introns may only be rare polymorphisms. Segregation analysis or functional analysis of the altered protein should be performed to evaluate the significance of missense mutations, including the two changes between similar amino acids [Val®Ala in Family 19 (BRCA1), Lys®Arg in Family 68 (BRCA2)], but no DNA samples from unaffected family members were available at this point.

Several recurrent *BRCA1* and *BRCA2* mutations have been reported in defined subgroups of certain populations, and these patterns eventually may be useful for development of cost-effective and routine methods for mutation screening and presymptomatic diagnosis. No specific or frequent mutations of *BRCA1* or *BRCA2* were detected among the Japanese families in this study. However, the 4-bp deletion (ACAG) of *BRCA2* found in two families (#18 and #50) was also found as a germline mutation in our previous study (Miki et al. 1996). This may be due to a hot spot for mutation or founder effect.

The average age of onset for breast and ovarian cancer in Japanese patients with constitutional *BRCA1* mutation was 51.8 years, that in patients carrying missense mutations was 50.2, and that in patients carrying nonsense or frameshift mutations was 52.7, as reported previously (Matsushima et al. 1995; Inoue et al. 1995; Katagiri et al. 1996b). The results in this study are similar to the previous data and indicate that the average of age of onset for breast cancer in Japanese patients with constitutional *BRCA1* or *BRCA2* muta-

tions was much later than in the case of predisposed patients in the United States or western Europe (Kelsey and Berkowitz 1988). It is notable, however, that the average onset age for breast or ovarian cancer in our panel of patients with missense mutations was similar to that in patients with nonsense or frameshift mutations, even though the missense mutations predominated. These results imply that other factors such as hormonal or nutritional differences among ethnically or geographically separate populations influence the time of cancer onset among predisposed individuals.

Acknowledgments We thank Maiko Minami and Yumi Nakajima for their excellent technical assistance, and Drs. Moritoshi Kinoshita and Takafumi Fukui (Otsuka Assay Laboratory, Otsuka Pharmaceutical Co., Ltd.) and Dr. Mitsuru Emi (Nippon Medical School) for their contributions to the mutational screening of *BRCA1*. We are grateful to The Japanese Familial Tumor Society and The Japanese Breast Cancer Society for the identification of families. This work was supported in part by a special grant for Strategic Advanced Research on Cancer from the Ministry of Education, Culture, Sports, and Science of Japan and by a grant from the Japanese Ministry of Health and Welfare.

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