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Mutation analysis of the *BRCA1* gene in 76 Japanese ovarian cancer patients: four germline mutations, but no evidence of somatic mutation

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To investigate the putative role of *BRCA1*, a gene involved in hereditary breast and ovarian cancer, in sporadic ovarian tumors among Japanese women, we examined 76 unselected primary ovarian cancers for mutations in the coding region of *BRCA1* using the single-strand conformation polymorphism technique. Although no somatic mutations were detected in any of the tumors, constitutional mutations were identified in four cases: two frameshifts, one nonsense mutation and one intronic base substitution 32 bp downstream of exon 22; RT-PCR experiments revealed that the single-base substitution in the intron seemed to increase the transcript lacking exon 22. All four cases were judged to involve truncation of the gene product. The evidence reported here supports a rather limited role of *BRCA1* in ovarian carcinogenesis in the Japanese population.

INTRODUCTION

BRCA1, a gene involved in inherited predispositions to breast and ovarian cancers, was recently isolated (1,2). Germline mutations in *BRCA1* are thought to be responsible for ~45% of inherited breast cancers and for >80% of breast and ovarian cancers arising in families that exhibit a high incidence of both types of tumor (3). Since publication of the first two manuscripts describing five *BRCA1* mutations in eight affected families and four germline mutations in 44 sporadic ovarian or breast tumors, dozens of germline mutations of this gene have been identified (4–9).

In view of the markedly high incidence of ovarian cancer in families carrying a 17q-linked predisposition to breast cancer and the fact that loss of heterozygosity (LOH) at chromosome 17q, including the *BRCA1* region, is observed in 30–70% of sporadic breast cancers examined as well as in sporadic ovarian cancers (10–15), *BRCA1* is thought to be related to development of both types of cancer. Hence, by analogy with other tumor suppressors, investigators have expected to find frequent somatic mutations of the *BRCA1* gene in sporadic forms of breast and ovarian cancers. However, up to this point, all mutations found in breast cancers have been germline mutations, although Merajver *et al.* (8) detected somatic mutations in one allele of *BRCA1* in four sporadic ovarian tumors in which the other allele had been lost. Their data suggested that somatic inactivation of *BRCA1* may be implicated in a small proportion of sporadic ovarian tumors.

To further confirm those results, we screened tumor DNAs for *BRCA1* mutations in 76 unselected cases of ovarian cancer. Here we report detection of four germline mutations in this group of patients, three in the coding region and one within an intron. All four mutations would be expected to result in loss of function.

RESULTS

The entire coding sequence of the *BRCA1* gene, including exon–intron boundaries, was screened by PCR–single-strand conformation polymorphism (SSCP) analysis of DNAs from 76 ovarian tumors. We detected SSCP-variant bands in four samples (tumors 16, 24, 27 and 70) and subsequently determined nucleotide sequences of these tumor DNAs and corresponding constitutional DNAs by direct sequencing of PCR products. The results disclosed two frameshift mutations and one nonsense mutation in the coding region and one single-base substitution within an intron (Table 1).

In patient 16, a 1 bp insertion at codon 23 had created a new stop, immediately downstream, that would truncate the gene product (Fig. 1a). Patient 27 had a 4 bp deletion at codon 731; deletion of four nucleotides (AAGA or AGAA) began at the AAAGAA sequence of codons 731 and 732. This mutation also leads to premature termination of the *BRCA1* reading frame immediately downstream and would produce a truncated protein lacking nearly half of the COOH portion (Fig. 1b). A nonsense mutation from Arg to termination was observed in patient 70, in whom a C-to-T substitution had occurred at the first nucleotide of codon 1203 (Fig. 1c).

In patient 24, an A-to-T substitution had occurred in the intronic sequence 32 bp downstream of the last nucleotide of exon 22 (Fig. 1d). To investigate whether this substitution represented a rare polymorphism or, alternatively, would affect the splicing of the transcript, we performed reverse transcript-polymerase chain reaction (RT-PCR) using RNA prepared from the normal tissue of this patient. cDNA synthesized from

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Table 1. Germline mutations of the BRCA1 gene in ovarian tumor patients

Patient no.	Exon	Codon	Mutation	Nucleotide change	Effect on coding sequence	Effect on protein
16	2	23	185insA	1 bp insertion GAG→AGAG	Frameshift	Truncation at codon 40
27	11	731	2311del14 or 2312del4	4 bp deletion AAAGAA→AA	Frameshift	Truncation at codon 734
70	11	1203	Arg1203ter	CGA→TGA	Nonsense	Truncation at codon 1203
24	Intron 22		5526 + 32 A→T	A→T at 5526 +32	Aberrant splicing	Truncation at codon 1804

The BRCA1 mutations were designated according to a suggested nomenclature (17). Nucleotides were numbered according to the sequence of GenBank accession no. 14680. Codon numbers were assigned earlier (1).

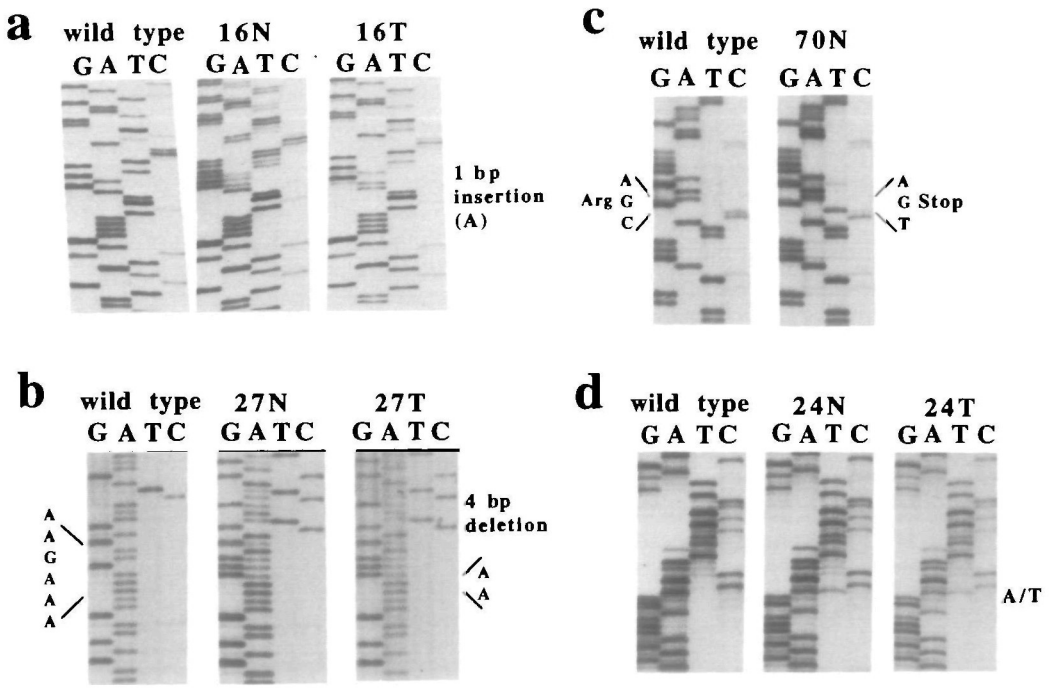

Figure 1. Sequence analyses of PCR products of tumor DNAs and corresponding normal DNAs from the four patients whose tumor DNAs revealed aberrant bands by SSCP. Constitutional (N) and tumor (T) DNAs each revealed mutated DNA sequences. (**a**) A 1 bp insertion at codon 23 in Patient 16. (**b**) A 4 bp deletion at codon 731 in Patient 27. (**c**) A nonsense mutation of Arg to stop at codon 1203 in Patient 70. (**d**) A one-base substitution of A to T in intron 22. Wild-type sequences from unrelated, normal chromosomes are included as controls.

mRNA was amplified with primers corresponding to the DNA sequences of exons 20 and 24. Electrophoresis of the PCR product in a polyacrylamide gel revealed a 287 bp fragment in addition to the normal 361 bp fragment and the intensities of the two bands were almost equal. Although RT-PCR experiments with Southern hybridization using RNAs isolated from several normal tissues (breast tissues from five individuals and liver tissues from 11 individuals) as templates also detected the 287 bp fragment, the 287 bp transcript was the minor product and the ratio of the 287 versus 361 bp bands was 1:20–1:30. Direct sequencing of the smaller fragment indicated skipping of exon 22 (Fig. 2a); in this product, all 74 bp of exon 22 were completely absent and exon 21 was directly connected to exon 23. This aberrant splicing would also cause a premature truncation of the gene product. All four mutations were confirmed by at least two additional, independent experiments; in cases where the base changes had destroyed or generated a cleavage site for restriction enzymes, digestion of the PCR product with appropriate endonucleases was also performed.

In all four patients, the mutations were carried in the constitutional (germline) DNAs and no somatic mutation was found in any of the other 72 tumors either. As shown in Figure 1a, loss of the wild type alleles was detected in tumor DNA of Patient 16, indicating the two-hit mutation of *BRCA1*. Three of these tumors [16,27,70] were serious cystadenocarcinomas and one [24] was mucinous cystadenocarcinoma. The ages of onset of these patients were 51 [16], 44 [24], 56 [27] and 46 [70] years old.

A retrospective review of medical records revealed that two of the patients carrying mutant alleles of *BRCA1* had relatives with ovarian cancer: the mother and a younger sister of Patient 27 each had developed ovarian cancer, at ages 55 and 45 respectively; in addition to her ovarian cancer, Patient 70 had

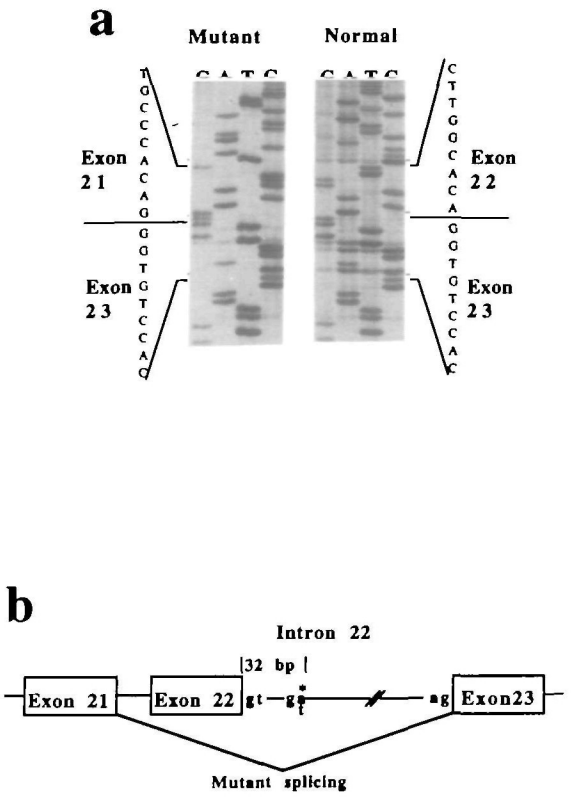

Figure 2. Aberrant splicing caused by an intronic base substitution. (**a**) DNA sequencing of the RT-PCR product from Patient 24. Wild-type cDNA sequence from the normal electrophoretic band and mutant cDNA sequence from the faster migrating band are shown. (**b**) Schema of this region. A-to-T substitution leads to aberrant splicing of exon 21 to exon 23. The whole of exon 22 is skipped in the variant.

developed bilateral breast cancers at ages 45 and 53 and a paternal cousin had developed a breast cancer at age 45 and an ovarian cancer at age 65. Patients 16 and 24 had no family history of breast and/or ovarian cancers.

DISCUSSION

From the evidence that dozens of constitutional mutations in *BRCA1* have been identified in breast cancer patients, it is now certain that germline mutation of this gene is the primary predisposing factor in families carrying 17q-linked predispositions to breast and ovarian cancers. About 70% of the mutations so far detected have been frameshifts and 10% nonsense mutations. The reported mutations are scattered throughout the *BRCA1* gene and no hot-spot has been identified.

Loss of heterozygosity (LOH) around the *BRCA1* locus is observed in 30–70% of sporadic breast and ovarian cancers (10–15). In 17q-linked families studied for LOH, the wild-type *BRCA1* allele has been lost in the tumors, suggesting that *BRCA1* acts as a tumor suppressor. If *BRCA1* in fact acts as a suppressor in both familial and sporadic forms of breast and ovarian cancer, somatic mutations of this gene should be detectable in sporadic tumors, most likely as a combination of a subtle mutation within one allele of the gene and a chromosomal deletion represented by LOH in the homologue. Marajver *et al.* (8) reported somatic mutations in some ovarian cancers in conjunction with LOH for an intragenic *BRCA1* marker, but the frequency was very low (9%). Furthermore, no somatic mutation has yet been reported in any breast cancers.

We detected germline mutations in four (5.2%) of 76 patients with ovarian tumors. Although our study included a larger number of tumors than the Merajver study, we found no somatic mutations. The reason for this difference ($P = 0.017$, by Fisher's exact test) is unclear; it could be due to environmental differences affecting the two populations or it could reflect a lower sensitivity of our screening method. However, as we were able to find germline mutations by SSCP, the latter possibility seems unlikely. Our results indicate that somatic mutation of *BRCA1* may play only a minimal, if any, role in the pathogenesis of sporadic ovarian cancers in Japanese women. Furthermore, the mean age of onset among the four Japanese patients with constitutional *BRCA1* mutations was 49 years, similar to that of the 72 other patients examined in this report. Similarly, screening for *BRCA1* mutations in >500 breast carcinomas (our unpublished data) has revealed a generally later onset (50 years) of breast cancer in Japanese carriers of mutant *BRCA1* alleles than has been reported in the USA or Western Europe.

Aberrant splicing of this gene has been described by others (4), in which a T-to-G substitution generated a cryptic acceptor site that resulted in insertion of 59 nucleotides of intronic sequence into the transcript. It is interesting that one of the four cases reported here had a one-base substitution 32 nucleotides downstream of an exon–intron boundary, possibly increasing an efficiency of aberrant splicing that skipped one 5′ exon. Although some alterations in intron 21 may have caused this splicing, at least 100 bp each of 5′-intronic region of exon 22 and 3′-intronic region of exon 21 was also examined by SSCP, but no alteration was detected. The great majority of intronic mutations that are known to cause dysfunction of cancer-associated genes or genes associated with hereditary diseases, except the cases that affect consensus splicing-acceptor (GT) or -donor (AG) sites, generate cryptic donor or acceptor sites. However, our RT-PCR experiment clearly proved that the intronic base substitution in Patient 24 caused skipping of the exon immediately upstream. This unusual case may be an important resource for studying the splicing machinery.

MATERIALS AND METHODS

Ovarian cancer patients

Specimens were obtained from 76 unselected patients who underwent surgery for ovarian cancer between 1990 and 1995 at the Cancer Institute Hospital, Nagoya City University Hospital, Sapporo Medical School Hospital, or Kinki University Hospital. The average age at diagnosis was 53 years (range: 17–87 years). Pathologic types of tumors were as follows: serous (40/76), clear cell (17/76), mucinous (13/76), endometrioid (1/76), transitional cell (1/76), mixed epithelial (1/76), Brenner (1/76) and undifferentiated (2/76). The stage was known for 54 (71.1%) of the tumors; 35.2 % of the stage-known tumors were diagnosed at stage I, 11.1% at stage II, 48.1% at stage III and 5.6% at stage VI.

A retrospective review of the clinical records revealed that five of the 76 patients [20,48,57,70,74] had also developed a malignancy in the breast. Four patients [21,27,68,73] had multiple family members affected with ovarian cancer and one patient [70] had a familial history of breast and ovarian cancer. All others were sporadic cases.

Mutation analysis

DNA samples were extracted from tumors and corresponding noncancerous tissues by standard methods. Thirty-three DNA segments representing the entire coding region of *BRCA1* were amplified separately by polymerase chain reactions. Primer sequences were presented in a previous paper (9). For exon 11, which is 3245 bp long, PCR primers were designed to amplify eleven overlapping segments of this exon separately. Each of the other 22 exons was amplified individually in a single PCR.

Genomic DNA (50 ng) was amplified in a reaction mixture containing 2.5 pmol of each primer, 0.2 pmol of each dNTP, 2 μCi of [α-^{32}P]-dCTP (3000 Ci/mmol, 10 mCi/ml) and 0.25 units of *Taq* polymerase in 10 μl of amplification buffer. Cycling conditions were 35 cycles of 94°C, 30 s, 58°C, 30 s, 72°C, 30 s. The reaction mixture was diluted with 95% formamide dye, incubated at 85°C for 5 min and applied to a 6% polyacrylamide gel containing 0.5% TBE (90 mM Tris–borate/2 mM EDTA) and 5% glycerol. Electrophoresis was performed at room temperature. The gel was dried and autoradiographed with an intensifying screen.

Direct sequencing

Genomic DNAs were amplified in a 100 μl PCR reaction as above, using 50 ng of tumor DNA and corresponding normal DNA. After purification on a 2% agarose gel, the PCR products were subjected to a 35 cycle asymmetric amplification under the same conditions, except for the use of limiting primers. The asymmetric PCR products were purified by selective precipitation to remove primers, using a spin column (UFC3, Millipore).

Single-stranded DNA produced by asymmetric PCR was sequenced by the dideoxy method with an inner set of primers. The single-stranded DNA and the primers were mixed in the annealing buffer and incubated at 68°C for 10 min and at 42°C for 5 min before addition of the sequencing reaction mix containing sequenase and ^{35}S dATP. After 5 min at room temperature, each mixture was divided into four parts and either ddATP, ddGTP, ddTTP or ddCTP was added to the individual reaction tubes. After 15 min incubation at 37°C, stop solution (95% formamide dye) was added. Samples were denatured at 80°C for 5 min and electrophoresed on 6% sequencing gels, which were then dried and autoradiographed.

RT-PCR

Total RNA from frozen non-cancerous tissue of Patient 24 was purified as previously described (16) and then reverse-transcribed by means of oligo(dT) priming and amplified with reverse transcriptase. The product was amplified by PCR, using an appropriate set of primers (5′-AGTCAGAGGAG-ATGTGGTCA -3′ in exon 20 and 5′-TATCAGGTAGGTGTCCAGCT-3′ in exon 24). Polymerase chain reaction products were electrophoresed on a polyacrylamide gel and stained with ethidium bromide. Bands were cut from the gel and used for direct sequencing.

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REFERENCES

1. Miki, Y., Swensen, J., Shattuck-Eidens, D., *et al.* (1994) A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science*, **266**, 66–71.
2. Futreal, P.A., Liu, Q., Shattuck-Eidens, D., *et al.* (1994) *BRCA1* mutations in primary breast and ovarian carcinomas. *Science*, **266**, 120–122.
3. Easton, D.F., Bishop, D.T., Ford, D., Crockford, G.P. and Breast Cancer Linkage Consortium. (1993) Genetic linkage analysis in familial breast and ovarian cancer. Results from 214 families. *Am. J. Hum. Genet.*, **52**, 678–701.
4. Friedman, L.S., Ostermeyer, E.A., Szabo, C.I., Dowd, P., Lynch, E.D., Rowell, S.E. and King, M.-C. (1994) Confirmation of *BRCA1* by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nature Genet.*, **8**, 399–404.
5. Simard, J., Tonin, P., Durocher, F., Morgan, K., Rommens, J., Gingras, S., Samson, C., Leblanc, J.-F., Belanger, C., Dion, F., Liu, Q., Skolnick, M., Goldgar, D., Shattuck-Eidens, D., Labrie, F. and Narod, S.A. (1994) Common origins of *BRCA1* mutations in Canadian breast and ovarian cancer families. *Nature Genet*., **8**, 392–398.
6. Castilla, L.H., Couch, F.J., Erdos, M.R., Hoskins, K.F., Calzone, K., Garber, J.E., Boyd, J., Lubin, M.B., Deshano, M L., Brody, L.C., Collins, F.S and Weber, B.L. (1994) Mutations in the *BRCA1* gene in families with early-onset breast and ovarian cancer. *Nature Genet.*, **8**, 387–391.
7. Shattuck-Eidens, D., McClure, M., Simard, J., *et al.* (1995) A collaborative survey of 80 mutations in the *BRCA1* breast and ovarian cancer susceptibility gene. *J. Am. Med. Assoc.*, **273**, 535–541.
8. Merajver, S.D., Pham, T.M., Caduff, R.F., Chen, M., Poy, E.L., Cooney, K.A., Weber, B.L., Collins, F.S., Johnston, C and Frank, T.S. (1995) Somatic mutations in the *BRCA1* gene in sporadic ovarian tumors. *Nature Genet.*, **9**, 439–443.
9. Katagiri, T., Emi, M., Ito,I., Kobayashi, K., Yoshimoto, M., Watanabe, S., Iwase, T., Kasumi, F., Miki, Y., Skolnick, M.H. and Nakamura, Y. (1995) Mutations in the *BRCA1* gene in Japanese breast cancer patients. *Hum. Mut.*, in press.
10. Eccles, D.M., Cranston, G., Steel, C.M., Nakamura, Y. and Leonard, R.C.F. (1990) Allele losses on chromosome 17 in human epithelial ovarian carcinoma. *Oncogene*, **5**, 1599–1601.
11. Jacobs, I.J., Smith, S.A., Wiseman, R.W., Futreal, P.A., Harrington, T., Osborne, R.J., Leech, V., Molyneux, A., Berchuck, A., Ponder, B.A.J. and Bast, R.C., Jr (1993) A deletion unit on chromosome 17q in epithelial ovarian tumors distal to the familial breast/ovarian cancer locus. *Cancer Res* , **53**, 1218–1221
12. Sato, T., Saito, H., Morita, R., Koi, S., Lee, J.H. and Nakamura, Y. (1991) Allelotype of human ovarian cancer. *Cancer Res.*, **51**, 5118–5122.
13. Lee, J.H., Kavanagh, J.J., Wildrick, D.M., Wharton, J.T. and Blick, M (1990) Frequent loss of heterozygosity on chromosomes 6q, 11, and 17 in human ovarian carcinomas. *Cancer Res.*, **50**, 2772–2728.
14. Cropp, C.S., Nevanlinna, H.A., Pyrhoenen, S., Stenman, U.-H., Salmikangas, P., Albertsen, H., White, R. and Callahan, R. (1994) Evidence for involvement of *BRCA1* in sporadic breast carcinomas. *Cancer Res.*, **54**, 2548–2551.
15. Futreal, P.A., Soederkvist, P., Marks, J.R., Iglehart, J.D., Cochran, C , Barrett, J.C. and Wiseman, R.W. (1992) Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. *Cancer Res.*, **52**, 2624–2627.
16. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
17. Beaudet, A.L. and Tsui, L.-C. (1993) A suggested nomenclature for designating mutations. *Hum. Mutat.*, **2**, 245–248.