Multiplex Mutation Screening of the \textit{BRCA1} Gene in 1000 Japanese Breast Cancers

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To detect \textit{BRCA1} mutations in Japanese breast cancer patients, we screened 1,000 unselected primary cancers for mutations in exon 11, which accounts for 61% of the entire \textit{BRCA1} coding sequence. Using a method based on multiplex single-strand conformational polymorphism (SSCP) analysis of multiple restriction fragments generated by restriction-enzyme digestion of amplified DNA, we identified eight mutations. All eight were germline mutations; four of them were non-sense mutations or small deletions resulting in premature stop codons, and the other four were missense mutations. The Japanese carriers of these mutant \textit{BRCA1} alleles had developed breast cancers at ages ranging from 45 to 62, five of them bilaterally.

Key words: \textit{BRCA1} — Familial breast cancer — Single-strand conformation polymorphism — Germ-line-mutation — Bilateral breast cancer

Statistical models formulated in epidemiological studies of breast cancer suggest that inherited susceptibility accounts for about 5% of all breast cancer cases in Caucasian women, and that approximately one in 200 women in the United States may carry such an inherited susceptibility, while the life-time risk of breast cancer is 1 in 9–10 among Caucasian women. Linkage studies in Caucasian families with a high incidence of breast and ovarian cancers, using polymorphic DNA markers, have shown that a locus at 17q21 accounts for an inherited susceptibility in about 45% of these families. Recently \textit{BRCA1}, a gene at 17q21 showing germline mutations in many cases of familial breast and ovarian cancers in Caucasians, was isolated by Miki et al. \textit{BRCA1} consists of 22 coding exons and encodes a protein of 1863 amino acids whose structure includes a RING-finger motif at the amino terminus. Subsequent studies have identified a number of different mutations within this gene in patients with Caucasian hereditary breast cancers. In Japanese breast cancer families, Inoue et al. found two \textit{BRCA1} mutations and three \textit{BRCA2} mutations in 20 families.

Because the results of such family-based studies may not represent the frequency, distribution, or nature of the \textit{BRCA1} mutation in a general clinic population, we chose to investigate a large number of primary breast cancers unselected for the presence or absence of family history of the disease. To this end, we used a new screening protocol to search for \textit{BRCA1} mutations among 1000 cases of breast cancer in Japan. We screened nearly two-thirds of the entire \textit{BRCA1} coding sequence using a method involving exon 11 of the \textit{BRCA1} gene. We amplified this 3425-bp exon as two DNA segments, each of which was then fragmented by digestion with multiple restriction enzymes and subjected to multiplex SSCP analysis. This method enabled us to screen our large test population rapidly and efficiently, and allowed us to detect \textit{BRCA1} mutations in the general clinic population of Japanese women.

MATERIALS AND METHODS

Patients and cancer specimens One thousand patients who underwent surgery for breast cancer at the Cancer Institute Hospital between September 1989 and December 1994 participated in this study. They included 103 high-risk patients genetically selected on the basis of family history, early onset or bilateral tumor, whom we have studied previously. DNAs were extracted from breast cancers and corresponding noncancerous tissues under protocols described previously.

Multiplex SSCP screening Exon 11 of the \textit{BRCA1} gene, which represents nearly two-thirds of the coding sequence, was first amplified as two fragments overlapping each other by about 50 bp. The 5-prime half of the exon was amplified as a 1910-bp fragment using primers IF (5’-CCAAGGTTATGAAGTATGAT-3’) and IR (5’-GAACTCTAAAGGAGCTTCT-3’), and the 3-prime half was amplified as a 1750-bp fragment using primers 7F (5’-GTTTTCCAAAGATAATG-3’) and 12R (5’-GAAGCA-
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GTTCCTTTAACTATA-3'). PCR were carried out in 10-μl volumes containing 500 ng of genomic DNA, 200 μM of each dNTP, 10 pmol of each primer, 1 μCi of [α-32P]dCTP and 0.5 units of Taq polymerase. Each sample was denatured at 95°C for 5 min, then PCR was conducted with 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products containing the 5′ half of exon 11 were digested into smaller pieces by using a combination of restriction enzymes EcoRI, XmnI, SstI, CfoI, HincII, and KpnI (2 units each) in 10 mM Tris (pH 7.4) containing 10 mM MgCl2, and 50 mM NaCl. The PCR product containing the 3′ half was digested by using a combination of PstI, HaeIII, Dral, and HincII. Locations of the PCR primers, amplified fragments, and restriction enzyme sites in each fragment are schematically shown in Fig. 1C. Each digested PCR product was mixed with 70 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 95°C for 5 min, and chilled on ice, then a 3 μl sample was applied to a vertical 30 cm x 40 cm x 0.2 mm polyacrylamide gel consisting of 6% polyacrylamide and 5% glycerol in 0.5x TBE buffer.13) Electrophoresis was carried out under three different conditions to improve resolution for detecting different types of SSCP variants: 300 V for 16 h at room temperature, 400 V for 16 h at 4°C, and 1200 V for 4 h at room temperature. Gels were dried and autoradiographed overnight with intensifying screens.

Regional mapping and sequencing of SSCP variants
Any SSCP variant detected in the multiplex SSCP analysis was further mapped to a smaller segment (average 350 bp in size) within the amplified region by conventional SSCP analysis using the primer sets and PCR protocols that we have described previously.11) For an SSCP variant detected in the 5′ half of exon 11, six PCRs were carried out, using primer set 11-a, 11-b, 11-c, 11-d, 11-e, or 11-f, separately. For an SSCP variant detected in the 3′ half of exon 11, five PCRs were carried out, using primer set 11-g, 11-h, 11-i, 11-j, or 11-k, separately. A PCR product that demonstrated an SSCP variation in the regional mapping was sequenced directly using gene-specific primers, or sequenced after subcloning, to identify the nature of the mutation. All results were confirmed in two independent experiments.

RESULTS

Representative autoradiograms of the multiplex SSCP analyses for the 5′ and 3′ halves of exon 11 are shown in Fig. 1. For each sample, the 5′ half was split into eight restriction fragments (the smallest 190 bp and the largest 335 bp), and analyzed by SSCP. The 3′ half was cut into six restriction fragments (ranging from 130 to 390 bp) and similarly subjected to SSCP analysis. We detected 12 to 16 bands that corresponded to sense or anti-sense single-stranded DNAs, depending on the electrophoretic conditions used.

Multiplex SSCP analysis revealed eight patients with aberrant SSCP-band patterns (Table I), four of whose muta-
tions had already been detected in an earlier, small-scale screening by conventional SSCP analysis (patients 23, 44, 98 and 100).11) The eight SSCP variants were each mapped into one of the 11 restriction fragments of exon 11 as shown in Fig. 2, and their nucleotide sequences were determined. The four novel mutations identified in the present study are shown in Fig. 3.

Constitutional and tumor DNAs of patient 1388 exhibited a 4-bp deletion (ATTA) at codon 482 of \textit{BRCA1}, resulting in a frame-shift and a premature stop codon downstream (1563delATTA). Patient 1446 had a T-to-C transition at the first nucleotide of codon 856, which would result in substitution of a histidine residue for the wild-type tyrosine at this site (Tyr856His). Constitutional and tumor DNAs of patient 1930 showed a G-to-A transition at the first nucleotide of codon 271, resulting in substitution of a methionine residue for valine at this site (Val1271Met). In patient 2264, the 1-bp deletion (T) at codon 852 would cause a frame-shift and premature stop codon downstream (2674delT).

All eight mutations, including the four identified previously, were present in the germline and no somatic mutation in the \textit{BRCA1} gene was found in any of the 1000 cases. Five of the eight mutations were found in patients who had developed cancers in both breasts (patients 23, 44, 98, 100, and 1930). Upon retrospective review of medical records, four patients in the present study were found to have family members affected with breast or ovarian cancers: the sister of patient 23 had developed breast cancer; the mother of patient 44 had an ovarian cancer and an aunt had a breast cancer; the mother of patient 1930 had a breast cancer; and the mother of patient 2264 had an ovarian cancer. Family background was unclear for the other four patients because medical records were incomplete.

DISCUSSION

To accomplish mutational screening of a 3425-bp genomic region in a large number of cases in a population-based study, we developed a method based on multiplex SSCP analysis of multiple fragments generated by restriction enzyme digestion of large PCR products. This method enabled us to screen nearly two-thirds of the \textit{BRCA1} coding sequence (represented by exon 11) by means of serial reactions in a single tube and in a simplified format.

The sensitivity of SSCP analysis depends on the length and the primary structure of the fragment to be analyzed and on the conditions chosen for electrophoresis. We digested the long PCR products with restriction enzymes into shorter fragments (190–335 bp in the 5’ half; 130–390 bp in the 3’ half) to achieve higher sensitivity. It is known that certain DNA variations are undetectable by SSCP under some electrophoretic conditions, but may become apparent under others. Michaud et al.\textsuperscript{14}) required three different sets of electrophoretic conditions in the SSCP analysis of the ornithine aminotransferase gene to detect all point mutations that had been characterized by other methods. To achieve a degree of sensitivity capable of detecting as many mutations as possible, we also carried out our electrophoretic experiments under three different sets of conditions.\textsuperscript{15)}

A predominance of frameshift or nonsense mutations resulting in truncated protein products appears to be characteristic of inherited \textit{BRCA1} anomalies; 51 of 63 mutations (81%) identified to date through complete screening of probands from high-risk families have belonged to this type.\textsuperscript{16} However, in our screening of breast cancer cases from a general clinic population, only half of the mutations found were frameshift or nonsense types and the other half were missense mutations. The three missense mutations reported here (the same one was observed in two cases) might represent very rare polymorphisms, but the absence of these alterations in the rest of the 1000 patients and 192 unaffected Japanese individuals makes this possibility unlikely.
Certain common mutations appear to be characteristic of alteration in the BRCA1 gene. In a recent compilation of data, 5382insC, 185delAG, and 4184del4 mutations were present respectively in seven, eight, and five of 63 probands with BRCA1 mutations. Eight other mutations were each found twice in that set of probands; thus, 11 recurrent mutations comprised 57% of those 63 mutations. In the present study, only one mutation occurred twice; a Val271Met missense mutation was found in patients 98 and 1930.

Among three American breast cancer patients reported to carry BRCA1 mutations who were unselected for family history, the ages of onset of breast cancer were 24, 39, and 42, consistent with the term “early onset.” In the present study, the age of onset in Japanese patients carrying germline BRCA1 mutations ranged from 45 to 71 (mean age 51), much later than in the United States.

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