Mutation analysis in the *BRCA2* gene in primary breast cancers

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Breast cancer, one of the most common and deleterious of all diseases affecting women, occurs in hereditary and sporadic forms. Hereditary breast cancers are genetically heterogeneous; susceptibility is variously attributable to germline mutations in the *BRCA1* (ref. 1), *BRCA2* (ref. 2), *TP53* (ref. 3) or ataxia telangiectasia (*ATM*)[4] genes, each of which is considered to be a tumour suppressor. Recently a number of germline mutations in the *BRCA2* gene have been identified in families prone to breast cancer[5,6]. We screened 100 primary breast cancers from Japanese patients for *BRCA2* mutations, using PCR-SSCP. We found two germline mutations and one somatic mutation in our patient group. One of the germline mutations was an insertion of an *Alu* element into exon 22, which resulted in alternative splicing that skipped exon 22. The presence of a 64-bp polyadenylate tract and evidence for an 8-bp target-site duplication of the inserted DNA implied that the retrotransposal insertion of a transcriptionally active *Alu* element caused this event. Our results indicate that somatic *BRCA2* mutations, like somatic mutations in the *BRCA1* gene, are very rare in primary breast cancers.

The *BRCA2* gene is composed of 27 exons distributed over roughly 70 kb of genomic DNA[6]. Using PCR-SSCP, we screened the entire coding sequence and intronic sequences flanking each of its exons for mutations in DNAs from 100 primary breast cancers. The DNA sequences of PCR products obtained from tumour DNAs, and corresponding constitutional DNAs corresponding to SSCP-variants, were determined.

We found one somatic and two germline mutations (Table 1). The tumour DNA from patient 1840 had a C-to-A transition at the first nucleotide of codon 2415 (Fig. 1*a*), which results in the substitution of asparagine for histidine. As this mutation was not present in the constitutional DNA, the alteration was considered to be a somatic event. In patient 1500, constitutional and tumour DNAs showed a 4-bp deletion (ACAG) in exon 9 (Fig. 1*b*), leading to a premature termination of *BRCA2* transcript due to a frameshift. In patient 2472, we found for exon 22 and its flanking sequences an approximately 650-bp PCR fragment in addition to the normal 300-bp fragment (Fig. 1*c*) This novel 650-bp fragment was also detected in the constitutional DNA of this patient. The 650-kb fragment was not detected in 100 control DNAs, suggesting that it was an insertion of about 350 bp into one of the *BRCA2* alleles of patient 2472.

To further characterize the insertion, we subcloned the 650-bp fragment and determined its nucleotide sequence. We found that the *BRCA2* gene was disrupted in exon 22 at base position 38 by insertion of 346 bp (Fig. 2*a*, *b*). A 64-base polyadenylate tract was found at the 3' end of the inserted sequence, and an 8-bp (TCACAGGC) target-site duplication of the *BRCA2* sequence (underlined in Fig. 2*a*) flanked the integrated

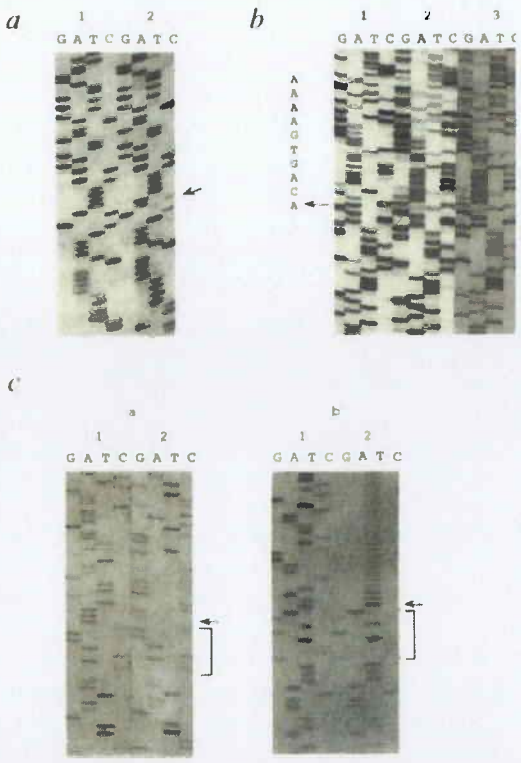

Fig. 1 Sequence analysis of PCR products of tumour and corresponding normal DNAs that showed aberrant bands by SSCP. *a*, A one-base substitution of C to A at codon 2415 (arrow) in tumour DNA (sequence 2) from patient 1840. This alteration was not present in the constitutional DNA (sequence 1). *b*, A 4-bp deletion (ACAG) in exon 9 found in constitutional (sequence 2) and tumour (sequence 3) DNAs from patient 1500. Sequence 1: normal control. The sequences of the normal and mutated alleles are indicated on the left and right of the panel, respectively. A box indicates the deleted nucleotides. *c*, DNA sequencing of the normal PCR product and the 650-bp fragment containing the insertion in patient 2472. Panel a, 5' end of the insertion (a2, arrow) and the normal counterpart (a1). Panel b, 3' end of the insertion (b2, arrow), preceded by polyadenylation, and the normal counterpart (b1). The 8-bp sequence in brackets (a2, b2) constitutes the presumed target-site duplication.

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Table 1 Mutations of the *BRCA2* gene in breast cancer patients

Patient No.		Exon	Codon	Nucleotide change	Effect of coding sequence	Age of onset
1840	Somatic	14	2415	CAT→AAT	Missense (His→Asn)	49
1500	Germline	9	252	4 bp deletion (ACAG)	Frameshift	34
2472	Germline	22	2934	346 bp insertion	Frameshift	65

None of the patients had a family history of breast cancer.

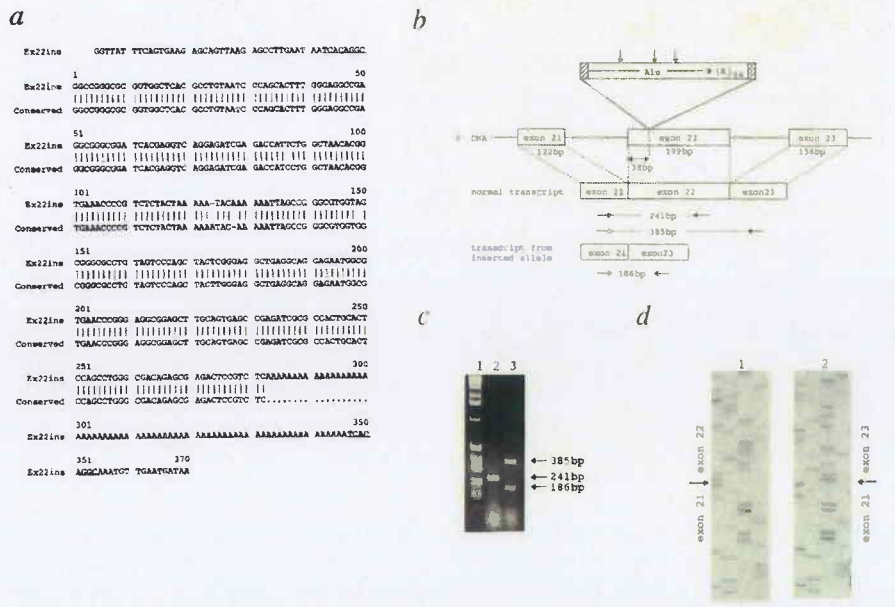

Fig. 2 Analysis of the insertional mutation in patient 2472. *a*, Comparison of the inserted sequence (Ex22ins) with *Alu* consensus sequences. The consensus sequence of the conserved *Alu* subfamily is on the lower ("Conserved") line. Vertical lines indicate nucleotide identity. Four nucleotides in the insertion are different from the consensus sequence of the *Alu* subfamily (at base positions 87, 124, 128, 149). Underlining indicates bases corresponding to the target-site duplication. *b*, Schematic diagram of the *Alu* insertion and the experimental design for RT-PCR analysis. Upper box indicates the location of the *Alu* insertion, flanked by an 8-bp target-site duplication indicated by hatched boxes. Vertical arrows denote sites of in-frame stop codons within the *Alu* insertion. Primers were selected to examine whether a transcript from the allele with the *Alu* sequence was present. Horizontal arrows under the "transcript" boxes indicate positions of DNA sequences corresponding to the primers for RT-PCR in that experiment. *c*, Agarose gel electrophoresis of RT-PCR products. Lane 1, DNA size markers(BRL 1-kb ladder); lane 2, product of a PCR using primers corresponding to DNA sequences in exons 21 and 22; lane 3, two products of a PCR using primers corresponding to DNA sequences in exons 21 and 23. *d*, Sequence analysis of RT-PCR products. Panel 1, DNA sequences of the exon 21–22 junction in the normal transcript; Panel 2, DNA sequences of the smaller transcript. Arrows indicate the boundaries. Comparison of these sequences revealed that abnormal transcript lacked exon 22 sequence, probably because of alternative splicing.

DNA without deletion of any *BRCA2* sequences. A search for homologies with DNA sequences in the public database revealed that the inserted fragment was highly homologous (different at only 4 of 282 nucleotides) to the consensus sequence of the conserved *Alu* subfamily[7,8] (Fig. 2*a*). We subsequently examined the *BRCA2* transcript in the same patient, considering it likely that inactivation of her *BRCA2* gene was caused by truncation of the gene product by an in-frame stop codon in the inserted *Alu* sequence (Fig. 2*b*). However, we found by RT-PCR amplification and subsequent DNA sequencing that the transcript lacked exon 22 (Fig. 2*c*, *d*). The insertion of the *Alu* sequence in exon 22, through some unknown mechanism, led to alternative splicing, causing this exon to be skipped.

Both the patients with germline mutations in *BRCA2* had no family history of breast cancer (Table 1). However, as the penetrance of Japanese patients carrying *BRCA2* mutation is unknown, it is uncertain whether these patients have *de novo* mutations. The position of the 4-bp deletion is located one nucleotide downstream of that described[6]. Although the number of the reported germline mutations in the *BRCA2* gene is very small, it may be possible that the region around codon 252 is a mutational hotspot.

Alu elements are interspersed repetitive sequences found in human DNA; they are mobile elements, with copy numbers in excess of 500,000 (ref. 9). Mobilization of *Alu* elements is thought to occur through an RNA polymerase III-derived transcript in a retroposition process[10]. The *Alu* element inserted in the *BRCA2* allele reported here is flanked by perfect 8-bp duplications of the target site without deletion of the original *BRCA2* gene sequence; this phenomenon is characteristic of insertions of mobile elements into staggered single-strand nicks[11]. Our results indicate that the *Alu* insertion may have been integrated by retrotransposal events. The *Alu* insertion into the *BRCA2* gene would be expected to alter the gene product significantly, as it resulted in the skipping of exon 22 (199 bp) in the transcript; this event would shift the coding frame and lead to an early termination of translation. Retrotransposal integration of sequences such as *Alu* and *LINE-1* into biologically important genes is thought to play a significant role in several human genetic diseases[12–16]. For example, we reported disruption of the *APC* gene by retrotranscriptional insertion of *L1*[17]. It will be of interest to determine the frequency with which transposable element-mediated inactivation of human genes occurs in somatic or germline cells, and to discover whether inherited or environmental factors influence that frequency.

The present demonstration of two novel constitutional mutations in *BRCA2* adds to the published evidence that germline mutation of this gene is the primary predisposing factor in some families prone to breast cancer. However, we found only one somatic mutation of *BRCA2* among the 100 primary breast cancers examined. A similarly low frequency characterizes mutations of the *BRCA1* gene: dozens of germline mutations of *BRCA1* have been identified in breast cancer patients, but to date no somatic mutations of that gene have been reported in any breast cancers. As a result, any role that *BRCA1* may play in sporadic breast and ovarian cancers remains elusive. Recently, Holt *et al.*[18,19] presented evidence that *BRCA1* is a selective growth inhibitor of breast and ovarian cells, a tumour suppressor gene. It is not known whether *BRCA1* and *BRCA2* function in the same pathway of tumour suppression. One possible explanation for the low frequencies of somatic mutations in *BRCA1* and *BRCA2* in primary breast cancers is that their transcription and/or translation may be regulated by a target gene that is more favored for mutation in sporadic tumours than either of them. The decreased expression of *BRCA1* in sporadic breast cancers[20] supports this hypothesis. We have not examined the expression of *BRCA2* in sporadic tumours, but our results suggest that somatic mutation of *BRCA2* is not a major contributor to carcinogenesis in sporadic breast cancers.

Table 2 Sequence of *BRCA2* primers used for PCR-SSCP analysis

Exon	Sense primer (5'→3')	Antisense primer (5'→3')
2	CTCAGTCACATAATAAGGAATGC	CAACACTGTGACGTACTGGGT
3	CAAATTTGTCTGTCACTGGTTA	CTAAATTCCTAGTTTGTAGTTC
4	CAAAGAATGCAAATTTATAATCC	CATCTTTATAGTTCAAATATATGTA
5	ATATCTAAAAGTAGTATTCCAACA	AAACTCCCACATACCACTGG
6	CTACAATGTACACATGTAACAC	AATCTCAGGGCAAAGGTATAAC
7	CGTTAAGTGAAATAAAGAGTGAATGA	TAACAGAATTATTAGAGATGACAATT
8	GTGTCATGTAATCAAATAGTAGATGT	AATGTAAGATAAATAATTTAACAAGG
9	TACTACTATATGTGCATTGAGA	ACAGAGCAAGACTCCACCT
10	TAGCACATTCTACATAAACTGTTC	CACAGAAGGAATCGTCAGCTA
11-A	TTTAGTGAATGTGATTGATGGTA	GTAAATGTGCAGATACAGTATTA
11-B	TTGTAAATACCTTGGCATTAGA	GTCCCTGGAAGGTCACTAGT
11-C	TGGACATTCTAAGTTATGAGGAA	ACTTTCTCCAATCCAGACATAT
11-D	CTCTAGATAATGATGAATGTAGC	CTTAATTGTTAGCATACCA
12	AAAATGGTCTATAGACTTTTGAG	ACCTATAGAGGGAGAACAGAT
13	ACAGTAACATGGATATTCTCTTA	AAACGAGACTTTTCTCATACTG
14	CTGCAACAAAGGCATATTCCTAA	ATATCTAACTGAAAGGCAAA
15	ATTTAATTACAAGTCTTCAGAATG	ATAAAAGCCATCAGTATTGTAG
16	TTTATTGTGTGATACATGTTTACT	AAAGAGGGATGAGGGAATAC
17	GTTGAATTCAGTATCATCCTAT	ATAGGATGATACTGAATTCAAC
18	CTTGTTTAAACAGTGGAATTCTA	TAACTGAATCAATGACTGAT
19	GAATTGAATACATATTTAACTACTA	CCATCTCAAACAAACAAACAAAT
20	ACTGTGCCTGGCCTGATAC	TGTTAAATTCAAAGCCTCTAAGA
21	TATGCTTGGTTCTTTAGTTTTAG	CTCACCTTGAATAATCATCAAG
22	GTTCTGATTGCTTTTTATTCC	AGTAAGGTCATTTTTTAAGTTAAT
23	TTTAAATGATAATGACTTCTTCC	TCCATAAACTAACAAGCACTTAT
24	TTTATGGAATCTCCATATGTTGA	CTGGTAGCTCCAACTAATCAT
25	CTTAAAATTCATCTAACACATCTA	AAAAATACCAAAATGTGTGGTGA
26	ACATAAATATGTGGGTTTGCAAT	ACGATGGCCTCCATATATACT
27	GAGACTGTGTGTAATATTTGCGT	AATAAAGCAGGCAGAATCA

Methods

Samples. Tumour and corresponding normal tissue were obtained at surgery from 100 breast cancer patients, 12 of whom reported positive family histories of breast cancer. We obtained the informed concent for genetic study from these patients.

Mutation analysis. Entire exons and their associated splice junctions were examined by PCR-SSCP. Primers used for PCR-SSCP are listed in Table 2. Genomic DNA (10 ng) was amplified by PCR; conditions consisted of 1 cycle at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 1 cycle at 72 °C for 2 min. Reactions took place in 10-μl volumes of 1× PCR buffer (25 mM TAPS, 50 mM KCl, 2 mM $MgCl_2$ and 1 mM beta-mercaptoethanol) containing 5 pmole primers, 20 μM dNTPs, 0.5 U *Taq* polymerase, and 2 μCi of [α]^{32}P-dCTP (3,000 Ci/mmol, 10 mCi/ml). Each reaction mixture was incubated at 85 °C for 5 min and electrophoresed in a 6% polyacrylamide gel containing 5% glycerol at 16 °C. When variant bands were revealed in SSCP analysis, the PCR products of tumour and corresponding normal DNAs were electrophoresed on 2% agarose, extracted from the gel, and subcloned into pT7-Blue (Novagen). Nucleotide sequences were determined in the subclones by dideoxy-chain termination with T7 DNA polymerase, using sequences nested in the PCR primers.

Multiplex SSCP analysis. A multiplex SSCP technique was used to screen exons 10, 11, 14, 18, and 27 as each of these exons was longer than 350 bp. PCR was carried out under the same conditions as above except that extension was performed for 2 min. PCR products were digested by various combinations 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, *Hin*dIII for exon 11-D; *Dra*I for exon 14; *Sau*3AI for exon 18; and *Dra*I, *Msp*I, *Sca*I, *Bcl*I for exon 27. Digested PCR products were electrophoresed in 6% polyacrylamide gels containing 5% glycerol.

RT-PCR analysis. Total RNA was extracted from normal tissue of the patient with ISOGEN (Nippon Gene), a procedure based on acid guanidine thiocyanate-phenol-chloroform extraction[21]. Reverse transcription was carried out as described[22], using 100 ng of total RNA. PCR was performed under the same conditions as the genomic PCR. Primers in exons 21, 22, and 23 were 5'–GTGCACTAACAAGACAGCAA–3', 5'–TTGTGACATCCCTT–3', and 5'–TAGATTTTGAAGTTGCAAGATG–3' respectively. RT-PCR products were subcloned, then sequenced using nested primers (5'–TGCAAGATGGTGCAGAGCTT–3' for exon 21, 5'–CAGATTCCATGGCCTTCCTA–3' for exon 22, and 5'–TTCTGTATCTCTTTCCTTCTG–3' for exon 23).

Sequence accession number. The sequence data will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence data bases under accession number D83989.

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