BRCA2 mutations in primary breast and ovarian cancers

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The second hereditary breast cancer gene, *BRCA2*, was recently isolated[1]. Germline mutations of this gene predispose carriers to breast cancer, and, to a lesser extent, ovarian cancer. Loss of heterozygosity (LOH) at the *BRCA2* locus has been observed in 30–40% of sporadic breast and ovarian tumours, implying that *BRCA2* may act as a tumour suppressor gene in a proportion of sporadic cases [2–5]. To define the role of *BRCA2* in sporadic breast and ovarian cancer, we screened the entire gene for mutations using a combination of techniques in 70 primary breast carcinomas and in 55 primary epithelial ovarian carcinomas. Our analysis revealed alterations in 2/70 breast tumours and none of the ovarian carcinomas. One alteration found in the breast cancers was a 2-basepair (bp) deletion (4710delAG) which was subsequently shown to be a germline mutation, the other was a somatic missense mutation (Asp3095Glu) of unknown significance. Our results suggest that *BRCA2* is a very infrequent target for somatic inactivation in breast and ovarian carcinomas, similar to the results obtained for *BRCA1*.

We analysed genomic DNA from 70 breast and 55 epithelial ovarian cancers for *BRCA2* mutations. Thirty-four of the breast and 18 of the ovarian tumours (56% and 53% respectively of informative cases) showed LOH in the *BRCA2* region, using the markers *D13S260, D13S171, D13S260,* and *D13S153*. The latter marker is intragenic to the *RB1* gene. All tumours analysed which were informative for both the *BRCA2* and the *RB1* regions of 13q show concomittant LOH (data not shown). The complete coding region was screened in each sample using both single strand conformation analysis (SSCA) and denaturing gel deletion analysis. In addition, exons 10, 11 and 27 were also screened by the protein truncation test (PTT). Potential sequence alterations represented by aberrant bands were characterized by direct sequencing in all cases.

Table 1 ***BRCA2*** **mutations in primary breast cancer**

Patient no.	Age	Mutation	Effect	Germline/ somatic
19	59	4710delAG	frameshift	germline
6	74	C to A	Asp3095Glu	somatic

Sequence alterations were identified in 2/70 breast tumours (Table 1), but none of the 55 ovarian samples. An SSCA shift in exon 11 was detected in breast carcinoma sample #19 (Fig. 1). This fragment, along with the corresponding fragment from the patient's normal lymphocyte DNA and a healthy control, were sequenced directly. A 2-bp AG deletion was detected, corresponding to a 4710delAG mutation. This deletion produces a frameshift leading to a predicted premature termination codon 2 amino acids downstream. The deletion was present in the patient's germline (Fig. 1*b*). No family history of cancer had been reported by this individual, who was diagnosed at age 59 with a unilateral infiltrating ductal carcinoma. In breast carcinoma sample #6 a somatic alteration was detected. This alteration was found to be a C to A transversion in exon 25 resulting in an aspartate to glutamate amino acid change at codon 3095 (Fig. 2). The change was not present in the patients lymphocyte DNA, nor in over 300 control chromosomes. Six different highly polymorphic microsatellite markers on chromosomes 16 and 17 confirmed that the tumour and lymphocyte DNA came from the same patient (data not shown). This tumour sample had LOH in the *BRCA2* region and it was the aberrant glutamate residue that was retained in the tumour. This patient had a unilateral infiltrating ductal adenocarcinoma diagnosed at age 74. The significance of this amino acid change is unclear. Additionally, several sequence variants were detected via SSCA[6].

The lack of *BRCA2* mutations in sporadic breast and ovarian cancers is very reminiscent of the results obtained for *BRCA1*[7–10]. Similar to the observation for *BRCA1*, the region containing *BRCA2* undergoes LOH in a fraction of breast and ovarian carcinomas[8]. The

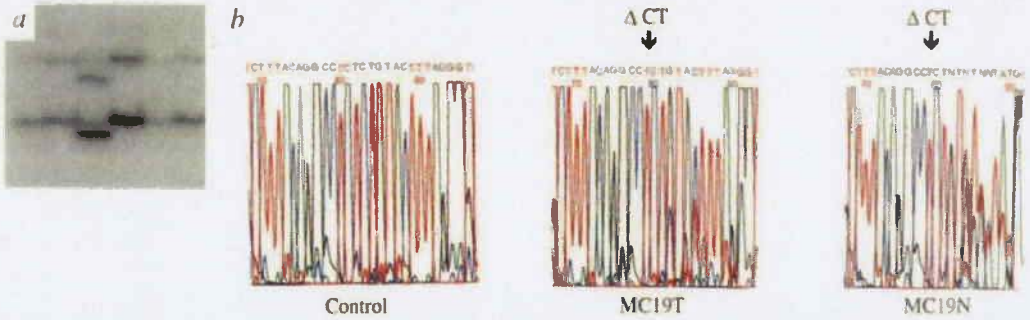

Fig. 1 *a*, SSCA autoradiogram showing shift in Patient 19 in lane 3. *b*, ABI electropherograms showing germline delAG in patient 19. The antisense strand is shown. MC19T, DNA from patient's tumour. MC19N, DNA from patient's peripheral blood. Control is unrelated normal blood DNA.

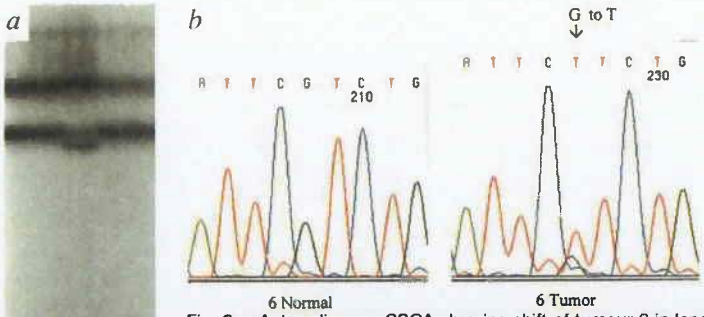

Fig. 2 *a*, Autoradiogram SSCA showing shift of tumour 6 in lane 3. *b*, Electropherogram showing sequence of missense mutation in tumour 6. Antisense strand is shown for both patients' tumour and normal blood DNA. The G to T transversion in tumour DNA shown in the right panel results in a GAC to GAA/ Asp to Glu amino acid substitution on the sense strand. This missense change was not present in the matching peripheral blood DNA from the same patient in the left panel.

Table 2a *BRCA2* PCR primers

Exon	Primers	Sequence (5'→3')
2	JAD46	F-CAAGCATTGGAGGAATATCG R-GTTTATGGTTCTAAGCAACAC
3	JAD47	CACAAATTTGTCTGTCACTGG ATTGCATTACTTACCTAAGTC
4	JAD48	ATCCAGAGTATATACATTCTC GATCTTCTACCAGGCTCTTAG
5,6	JAD 52	AAAATAACCTAAGGGATTTGC CAAATTCTCAATTACTAAGTC
7	JAD49	CAATTCAGTAAACGTTAAGTG AACAGAAGTATTAGAGATGAC
8	JAD50	CATGTAATCAAATAGTAGATGTGC CTCAAAGGCTTAGATAAATTACAG
9	JAD51	GCATTGAGAGTTTTTATACTAGTG ACCTGTAGTTCAACTAAACAG
10	JAD54F JAD23R	GCTTCTGTTTTATACTTTAACAGG GATCAGTATCATTTGGTTCCAC
	JAD20F JAD23R	CAAAGACCACATTGGAAAGTC GATCAGTATCATTTGGTTCCAC
	JAD24	AAGCAAACGCTGATGAATGTG TGGTCACATGAAGAAATATGC
	JAD25	CAGGTCTAAATGGAGCCCAG GAGAAGTTCCAGATATTGCC
	JAD26	CATCTTGAATCTCATACAGAC GACATAAGGAGTCCTCCTTC
	JAD27F JAD20R	AAGCCTCTGAAAGTGGACTG GCAAATGTAAGTGGTGCTTC
11	JAD53	GATGGTACTTTAATTTGTCAC CAAGATCCTGAGAGATTACTG
	JAD22	GCTCTTTTGGGACAATTCTG ATAAAAGACTTTTCTGGGATTG
	JAD21	TGGAATACAGTGATACTGAC TTTTCAGGTGGCAACAGCTC
	JAD19	CCCATGGAAAAGAATCAAGATG GTTCCTTAGTATTCCTAAAGC
	JAD18	TGTCTTCCAAGTAGCTAATG CTGTGATTTGAAATTGGACC
	JAD17	ACATGAACAAATGGGCAGGAC TGGTTTGAATTAAAATCCTGC
	JAD16	GTCATATAACCCCTCAGATG CTGTACCTTCAAATTGCTTGC
	JAD15	CGATTGGTCAGGTAGACAGC CTCTGCAGAAGTTTCCTCAC
	JAD14	TGTTTCTACTGAAGCTCTGC GTTATCTTCATTTTCAGTATTTCTC
	JUL5 F3 JUL5 R4	TTGAAATGACTACTGGCAC CCTTCATAAACTGGCCAGATAAT
	JAD13	TGTCTTAAATTATCTGGCCAG AAATGACTCTTTGGCGACAC
	JAD12	AGATTTTGAGACTTCTGATAC TCCAGTACCAACTGGGACAC
	JUL5 F1 JUL5 R1	TGGACATTCTAAGTTATGAGG ATTTCACTAGTACCTTGCTCTTTT
	JAD11	TGATGAAAAAGAGCAGGTAC ACAAGGTTTTTATCATTATTG
	JAD10	CTGCCCCAAAGTGTAAAGAAAT AATGACTGAATAAGGGGACTGAT
	JAD9	TCCTGCAACTTGTTACAC GATTTTTGTCATTTTCAGC
	JAD8	AACCAGAAAGAATAAATACT CCTCAACGCAAATATCTTCAT
	JAD7	TTCCAAAGTAATATCCAATGTA ATTTTTGATTTATTCTCGTTGTT
	JAD6	AAGTGAAAGACATATTTACAGACAG TATGAAGCTTCCCTATACT
	JAD5	CACCTTGTGATGTTAGTTTG TTGGGATATTAAATGTTCTGGAGTA
	JAD4	AAGTAACGAACATTCAGACC CTGGGTTTCTCTTATCAACAGCA
	JAD3	GTCTTCACTATTCACCTACG AGTGAGACTTTGGTTCCTAAT
	JAD2	TTCAACAAGACAAACAACAGT GTCAGTTCATCATCTTCCATAAA
	JAD1	CTTACTCCAAAGATTCAGAAAACTAC AGCATACCAAGTCTACTGAATAAAC
12	JAD30	ATAAAACTGATATTATTTGCC TCCACCTGAGGTCAGAAT
13	S1237 S1236	TAAAGCCTATAATTGTCTCA CTTCTTAACGTTAGTGTCATT
14	A45 A44	AAGGAACGTCAAGAGATACAG GGTTGGTCTGCCTGTAGTAAT
	S1239 S1238	CTTCAAGCAATTTAGCAGTTTCAGG GCTGCTTGATTGGAGTTGTT
15	JAD31	ATTTAATTACAAGTCTTCAG ACTCTGTCATAAAAGCCATC
16	JAD32	TATTTTGTGTAGCTGTATACG AGGGAATACATAAAAGTTAAC
17	JAD33	ATTCAGTATCATCCTATGTGG TATGATTACGTAATGTAATGC
18	JAD34	GAATTCTAGAGTCACACTTCC ATCTAACTGGGCCTTAACAGC
	JAD35	TGGCCATTATTGAACTTACAG AATTGAGCATCCTTAGTAAGC
19	JAD36	GAATGAAAACTCTTATGATATCTG AAGAGACCGAAACTCCATCTC
20	JAD37	CACTGTGCCTGGCCTGATAC AGTCTCTAAGACTTTGTTCTC
21	JAD53	TAAATCTCCCTTCTTTGGGTG TTCCTTCTTGTGATGGCCAG
22	JAD38	TCTAGTTACAATAGATGGAAC AATCATTTTGTTAGTAAGGTC
23	JAD39	GCATCTTTCTCATCTTTCTCC TGAAATAAAATTTCATCTGAAAAC
24	JAD40	TTGTTAGTTTATGGAATCTCC TAATCATAAGAGATTTTAAAAGAC
25	JAD41	TTCCATTCTAGGACTTGCCC GTGGTGATGCTGAAAAGTAAC
26	JAD42	TTTATAAAGCAGCTTTTCCAC ATACTTCTTATAATATTCCTTGAG
27	JAD43	ACATAATTATGATAGGGCTACG AAATGTACAAATGGGACTAAC
	JAD44	AGCCTTGGATTTCTTGAGTAG TCCTAGTGGATTCACTGACAG
	JAD45	TCTTTTGTCTGGTTCAACAGG AAGCGTCAATAATTTATTGTC

relatively close proximity of the *RB1* gene could be driving the majority of the LOH seen in these cases. Our study set contained no tumours that demonstrated loss at one region versus the other when informative for both. However, for breast and particularly ovarian cancers, there is evidence to suggest that the *RB1* gene can be excluded from the regions of loss in some cases and is not always targeted for inactivation in cases with large scale losses on 13q (refs 3, 5, 11). Our screening set contained both tumours selected for LOH in the *BRCA2* region as well as unselected cancer cases to increase the likelihood of finding mutations. Additionally, we employed three complementary techniques in screening for mutations, and thus feel that few coding region mutations have been missed. The two alterations found were both from tumours showing LOH. One case was a germline frameshift deletion which was retained in the tumour, the other a somatic missense mutation which was also retained in the tumour. The significance of the latter somatic mutation is unclear. Our data showing infrequent mutations in sporadic tumours suggests that if *BRCA2* plays a significant role in tumourigenesis in the non-hereditary forms of these cancers, it is through a mechanism other than structural mutation. Further, combined with the data on somatic mutations of *BRCA1* in sporadic breast and ovarian cancers, the evidence suggests that hereditary breast cancer (and ovarian cancer in the context of breast/ovarian cancer syndromes) may be fundamentally different diseases at the molecular genetic level. This is not to suggest that these genes play no role in the development of non-hereditary breast and ovarian cancers. Recent evidence would suggest that subcellular localization and/or expression levels may be critical in *BRCA1* involvement in cancer[12,13]. Given the results presented here, parallel studies on the expression, localization and, ultimately, normal function of *BRCA2* are paramount.

Methods

Samples. Tumour tissue and matched blood lymphocytes were obtained from patients treated at Duke University Medical Center, USA, and the Royal Marsden Hospital, England and from the Gynecologic Oncology Group/Cooperative Human Tissue network ovarian tissue bank (USA). Tissues was obtained under general consent for discarded tissue and tumour/white cell banking. Genomic DNA was obtained from tumour tissue and blood using standard procedures. The breast cancers were all infiltrating ductal carcinomas with the exception of two pure intraductal carcinomas. The mean age of onset for the breast study set was 53. The ovarian carcinomas were of mixed histology, the majority being papillary serous (90%), and the mean age of onset for the tumours studied was 58. There were no cases of either bilateral breast or dual primary breast/ovarian cancer in the study set.

Single strand conformation analysis. The entire coding region, including intron/exon borders, was examined by SSCA (using primers given in Table 2*a*). Genomic DNA (20 ng) was amplified using primers under the following standard PCR conditions: 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$ (Promega), 0.2 mM dTTP, dCTP and dGTP, 0.05 mM dATP, and (α-P^{32})dATP (Amersham) at 1 μCi/reaction and 2 U *Taq* DNA Polymerase (Promega) in a final volume of 10 μl. Thermocycling conditions consisted of 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by one cycle of 3 min extension at 72 °C in a 9600 Thermocycler (Perkin-

Table 2b BRCA2 PTT primers

Exon	Primers	PTT primer sequence[a] (5'→3')
10	JAD20F × JAD20R	JL-PTTH GAAACAGTTGTAGATACCTCTGAAGA
11	JAD54F × JAD17R	JL-PTTD GATTCTGAAGAACCAACTTTGTCC
11	JAD17F × JAD14R	JL-PTTF GAAATCAAGCTCTCTGAACATAAC
11	JAD14F × JAD12R	JL-PTTE GAAACTTCTGCAGAGGTACATCCA
11	JAD12F × JAD8R	JL-PTTA GACATTCTAAGTTATGAGGA
11	JAD9F × JAD5R	JL-PTTB GGTCAACCAGAAAGAATAAATACT
11	JAD5F × JAD2R	JL-PTTG GGGAAGCTTCATAAGTCAGTC
27	JAD43F × JAD45R	JL-PTTJ TCTTCTCCTAATTGTGAGATA

[a]Each PTT primer is preceded by the T7/Kozak sequence 5'–GGATCCTAATACGACT-CACTATAGGGAGACCACCATG–3'.

Elmer). PCR product (4 µl) was diluted in 56 µl of loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 94 °C for 5 min, and rapidly cooled on ice. A sample of 4 µl was electrophoresed through a 0.5× MDE gel (AT Biochem) containing 0.6× TBE buffer, at room temperature for 14–18 h at 8 W, dried, and autoradiographed for 1–18 h. Migration shift analysis was also used for detection of small deletions/insertions[1]. Samples were amplified as described above and were run through 5% denaturing sequencing gels at 70 W, dried and autoradiographed for 1–4 h.

Protein truncation test. Exons 10, 11 and 27, which represent 64% of the coding region, were amplified in 8 segments by the polymerase chain reaction (PCR) using primers given in Table 2*b*. A 1 µl aliquot of each of these primary templates was subjected to 10 additional cycles of PCR, in a reaction mixture containing a modified primer carrying a T7 promoter and eukaryotic translation initiation sequence in place of the forward primer. A 20 µl reaction mix containing 6 µl of the secondary PCR product, 1.6 µl of ^{35}S-methionine (1,000 Ci/mmol, Amersham), 0.8 ul RNAsin (recombinant 40 U/µl), 0.4 µl TnT T7 RNA Polymerase, 0.2 µl amino acid Methionine(-) Mix, and 5.0 µl TnT Rabbit reticulocyte lysate (Promega), was incubated at 30 °C for 1 h. This product (6 µl) was electrophoresed on a 10–20% SDS-polyacrylamide Ready-Gel (Biorad), fixed, dried and autoradiographed for 3–18 h.

DNA sequence analysis. Sequencing templates were produced for samples showing aberrant mobility on SSCA or PTT. Aberrant SSCA bands were cut from the MDE gel and eluted in 100 µl of dH_20 for 90 min at 37 °C. An additional 30 cycles of PCR was then carried out to amplify the DNA eluted from the gel slice. A parallel PCR was carried out to amplify genomic DNA from the same patient using the same primers. PCR products were purified using the Wizard PCR Prep DNA Purification System (Promega) and sequenced using a PRISM DyeDeoxy Terminator Cycle Sequencing kit and a 373 automated fluorescent sequencer (Applied BioSystems), according to manufacturer's instructions.

Acknowledgements

We thank P. Biggs, N. Rahman and B. Gusterson for assistance. This work was funded in part by the NCI/Duke University Specialized Program of Research Excellence (SPORE) in breast cancer P50-CA68438, NIH/NCI grant R21-CA68348, NIH grant 1R21-CA66228, the National Insititutes of Environmental Health Sciences, the Cancer Research Campaign, BREAKTHROUGH Breast Cancer Charity and Jean Rook Appeal, the US Army, the Institute for Cancer Research. C.M.P. and C.L. were funded by the Swedish Cancer Foundation.

Received 28 February; accepted 29 March 1996.

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