Analysis of BRCA1 involvement in breast cancer in Indian women

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The involvement of the familial breast-ovarian cancer gene (*BRCA1*) in the molecular pathogenesis of breast cancer among Indian women is unknown. We have used a set of microsatellite polymorphisms to examine the frequency of allele loss at the BRCA1 region on chromosome 17q21, in a panel of 80 human breast tumours. Tumour and blood leukocyte/normal tissue DNA from a series of 80 patients with primary breast cancer was screened by PCR-amplified microsatellite length polymorphisms to detect deletions at three polymorphic BRCA1 loci. PCR-allelotype was valuable in examining allele losses from archival and small tumour samples. Loss of alleles at BRCA1 in the patient set, confirmed a noteworthy role of this gene in the molecular pathogenesis of breast cancer and was in accordance with its well-documented tumour suppressive function.

1. Introduction

Breast cancer although the second most common malignant condition in India as a whole, ranks number one in the city of Mumbai. The disease affects approximately 900 women in Mumbai annually and is responsible for an annual age-adjusted death rate of 30·1 per 100,000 (Notani 1997). *BRCA1* (the gene for breast cancer 1) localized on chromosome 17q21 (Hall *et al* 1990), is a tumour suppressor gene (Smith *et al* 1994; Friedman *et al* 1994; Neuhausen and Marshall 1994; Merajver *et al* 1995). It is responsible for both early onset breast and ovarian cancer, with 45% of breast cancer families and 70% of breast-ovarian cancer families linked to it (Easton *et al* 1993). Compared to other racial groups, the Indians are different with respect to their reproductive, dietary and environmental conditions. The present study is directed towards finding out the extent of BRCA1 involvement in the pathogenesis (rather than initiation) of breast cancer among Indian patients by assessing the rate of allele losses at this locus. This allelic loss is termed as loss of heterozygosity (LOH) and is demonstrated as a complete or partial reduction in the signal intensity of one of the two corresponding alleles in tumour DNA from patients heterozygous for a polymorphic marker. The term "deletion" is often used where LOH is observed regardless of the individual mechanism. In the presence of a mutated tumour suppressor gene, loss of the normal homologue unmasks the defective gene and allows unopposed dysfunction. A second somatic event causing loss of the normal homologue leads to tumorigenesis. This event is accompanied by a large-scale chromosomal deletion (Knudson 1971). The patient population includes cases of sporadic primary breast cancer, cases presenting with a family history of breast cancer and patients from the Parsi population. The Parsis are an ethnic Indian group presenting with a two and a half-fold higher relative frequency of this disease. They are known to have a westernized life-style and perhaps the differences in social, cultural and environmental conditions may reflect an ethnic variation in cancer occurrence in this group. Since family history is known to pose a two- to three-fold increase in the risk of breast cancer (Anderson 1992), this high-risk group is studied as a separate category. The effect of a gene with a cancer family syndrome on the pathogenesis of the disease in this group was thus studied separately.

2. Materials and methods

2.1 *Patient population*

Eighty patients diagnosed with primary breast cancer who belonged to one of the three following categories were included in this study.

2.1a *Sporadic breast cancer* (*n=40*): No cancer of any

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kind in the relatives of the index case as checked from medical records or personal correspondence.

2.1b *Familial breast cancer*: Where one first degree relative or two second degree relatives other than the index case were affected with breast cancer as checked from medical records or personal correspondence.

2.1c *Parsi cases of breast cancer*: This category included primary breast cancer cases from the Parsi community without any family history of the disease as confirmed from medical records or personal correspondence.

All these patients were treated at Tata Memorial Hospital, Parel, Mumbai. Each of these patients presented with unilateral breast cancer.

2.2 *Normal and tumour tissue samples*

For individuals of category (A), peripheral blood leukocytes were obtained from each patient with informed consent and used as a source of normal DNA, while they were being treated at the hospital. For individuals of categories (B) and (C), normal skin/lymph node paraffin sections served as the source of obtaining normal DNA. Paraffin-embedded breast tumours were used to extract tumour DNA for all three groups. Each of the sections of normal and paraffin-embedded tissues were checked microscopically by haematoxylin-eosin staining. All samples collected as sources of normal or tumour DNA were obtained from the patient, before any kind of chemo-, radio-, hormonal therapy was administered to her.

2.3 *DNA isolation and estimation*

DNA was extracted from peripheral blood lymphocytes (Sambrook *et al* 1989) or paraffin-embedded tissues (Brow 1990) as per standard protocols. The samples were checked by agarose gel electrophoresis, and their concentrations were estimated against a panel of densitometrically estimated l DNA standards.

2.4 *Polymerase chain reaction*

Polymesrase chain reaction (PCR) amplimers were generated using equal concentrations (50–100 ng) of genomic DNA from matched normal and tumour samples. dNTP (2·5 mM each) was used along with 0·2 µM of each of the two primers and 1 unit of Taq polymerase per 50 µl of reaction mix, 30–35 cycles of denaturation (94°C/1 min), annealing (58°C or 55°C/1 min) and elongation (72°C/1 min) were followed by a final elongation at 72°C/10 min. The PCR was standardized for each set of primers used in the study. The primers and their sequences are given in table 1.

2.5 *LOH analysis*

Fifteen µl of each PCR sample was loaded on a 15% polyacrylamide gel with 3 µl of DNA gel loading dye. The gel was run at a constant voltage of 150 V till the dye front reached the bottom of the plates.

2.6 *Silver staining*

A rapid silver staining method (Neilan *et al* 1994) was used to detect the bands. The gels were washed in deionized water and fixed in 10% ethanol. They were then shaken in 1% nitric acid and thoroughly rinsed in deionized water. Staining was continued in partial darkness, using a solution of 3% sodium carbonate and 0·05% formaldehyde until the desired band intensity was achieved. Development was stopped in 3% acetic acid and the gel was preserved in a solution of 10% ethanol and 5% glycerol.

Table 1. Microsatellite markers and their characteristics.

Locus	Primer	Sequence	Type	Annealing temp. (°C)	Average product size	No. of alleles	Hetero-zygosity (%)	Reference
EDH-17B	HSD-A3T	5′-CAG TAC TAA AGG CCC TAT TAT CAA A-3′ 5′-AGG CTG CAG TGA TTC CAG AT-3′	AAAT	58	209 bp	2	18	Friedman *et al* 1993 Anderson *et al* 1993
D17S1322	s754	5′-CTA GCC TGG GCA ACA AAC GA-3′ 5′-GCA GGA AGC AGG AAT GGA AC-3′	$(TTG)_{15}$	55	130 bp	7	67	Neuhausen *et al* 1994
D17S1323	s975	5′-TAG GAG ATG GAT TAT TGG TG-3′ 5′-AAG CAA CTT TGC AAT GAG TG-3′	$(TG)_{19}$	55	155 bp	6	44	Neuhausen *et al* 1994

3. Results

Each case was represented by two samples (normal and tumour) and was tested at each of the three BRCA1 loci for LOH. Thus a total of 480 amplicons were subjected to polyacrylamide gel electrophoresis. Frequencies of LOH in our series of breast cancers varied according to the locus tested. Tumours that were heterozygous (informative) for any given locus were analysed for allele losses. LOH was scored as a significant alteration in the relative allele intensities between the two alleles from the tumour samples compared to those from the normal as shown in the figure 1. The figure represents a typical LOH, showing loss of one constitutive allele in the tumour sample. To calculate the total loss at BRCA1 in each patient category, the number of tumours informative at one or more BRCA1 locus were noted. The percentage LOH at BRCA1 was then calculated as the per cent of informative tumours giving allele loss at any one of these loci. The results are as follows:

(i) *Sporadic cases*: Collectively out of the 40 sporadic tumours, 38 were informative at one or more loci and 23 of these (60·53%) presented with LOH at at least one BRCA1 locus. Moreover out of these 23 tumours, four presented losses simultaneously at more than one locus –two of them showed losses at HSD-A3T and D17S1322, whereas two others, at D17S1322 and D17S1323. Since the order of these loci are cen-EDH17B (HSD-A3T)-D17S1322-D17S1323-tel; it means that a large chunk of DNA in and around the gene are lost in these patients.

(ii) *Familial cases*: Collectively out of 20 tumours studied, 17 were informative at one or more loci and 7 out of these 17 (41·18%) presented with LOH at at least one BRCA1 locus. One case showed a loss simultaneously at both intragenic loci.

(iii) *Parsi cases*: Collectively out of 20 tumours studied, 16 were informative at one or more loci. Overall, 6 out of these 16 (35·71%) presented with LOH at any one BRCA1 locus.

(iv) *Total analysis of all cases*: Out of all 80 tumours studied, 71 were informative at one or more loci. Overall, 36 out of these (50·70%) presented with LOH at any one BRCA1 locus.

Individual analysis of LOH at each of the three loci in the different patient categories are represented in tables 2–6.

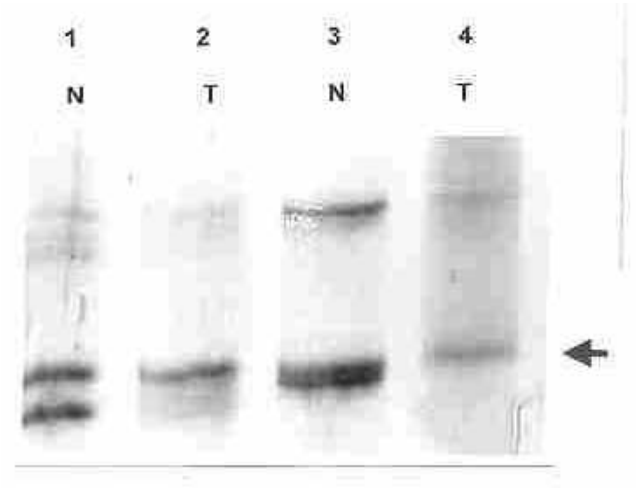

Figure 1. LOH at D17S1322. Normal (N) DNA and tumour (T) DNA from the same individual. Loss of one constitutional allele in tumour DNA is shown by the arrowhead.

4. Discussion

The extent of BRCA1 involvement in the pathogenesis of breast cancer among Indians is unknown. To the best of our knowledge, our study is the first to address this question. The patient population was subdivided into three groups with different risks of breast cancer. It is known that patients who have an affected first or second degree relative with breast cancer are known to present with a two- to three-fold increased risk of the disease (Anderson 1992). The Parsis, an ethnic community who settled in India about 1300 years ago from Persia represent a different study category for three reasons: (i) They show a higher accumulation of secondary risk factors, owing to their more westernized lifestyle. (ii) They have a conserved genetic pool, owing to a high frequency of consanguineous marriages. (iii) They present with a 2·5-fold higher incidence of breast cancer, compared to age matched patients from other Indian communities

Table 2. LOH in sporadic cases.

Locus	Number of tumours studied	Number of non-informative tumours	Number of informative tumours (x)	Tumours with LOH (y)	Percentage of tumours with LOH (y/x) 100
HSD-A3T	37	21	16	9	56·25
D17S1322	40	10	30	12	40·00
D17S1323	33	2	31	6	19·35

(Jussawalla *et al* 1985). The sporadic cases represent a low-risk group compared to the other two. This subdivision of patient samples was done to determine whether the extent of allelic loss at BRCA1 was different in different risk populations. However we did not see a higher rate of BRCA1 loss in the familial or Parsi cases compared to that seen in the sporadic cases. This suggests that the pathogenesis of breast cancer related to BRCA1 is the same irrespective of risk of developing the disease.

Allele losses previously reported in studies on sporadic tumours have ranged from 21–59% (Futreal *et al* 1992; Sato *et al* 1990; Cropp *et al* 1990, 1993; Kirchweger *et al* 1994; Futreal *et al* 1994) with a high value of 79% LOH reported at THRA1 (Futreal *et al* 1992). Another study reported an LOH of 50%, using two intragenic and one extragenic sequence tagged repeats (STRs) (Futreal *et al* 1994). A total LOH of 32% had been previously observed in a series of sporadic tumours at the intragenic BRCA1 locus, D17S855 (Dillon *et al* 1997). We report an overall loss of 60·53% in the BRCA1 region in the series of 40 sporadic tumours and losses of 18·6% and 40% at the two intragenic loci. A total of 43% LOH was seen at both these loci collectively. Moreover four samples gave large-scale deletions at this region suggesting deletion of all or part of the gene.

BRCA1 loss was seen to an extent of 41·18% in the familial cases. Studies over a span of four years have revealed that 16–45% of breast-ovarian cancer families have BRCA1 mutations (Easton *et al* 1993; Couch *et al* 1997).

We have found a cumulative LOH of 37·5% at BRCA1 in the 20 Parsi cases studied. Our estimates may be considered as the "minimum estimates" on BRCA1 LOH analysis, since our study has included three BRCA1 markers. This study

Table 3. LOH in familial cases.

Locus	Number of tumours studied	Number of non-informative tumours	Number of informative tumours (x)	Tumours with LOH (y)	Percentage of tumours with LOH (y/x) 100
HSD-A3T	19	13	6	3	50·00
D17S1322	20	13	7	5	71·42
D17S1323	19	5	14	1	7·14

Table 4. LOH in Parsi cases.

Locus	Number of tumours studied	Number of non-informative tumours	Number of informative tumours (x)	Tumours with LOH (y)	Percentage of tumours with LOH (y/x) 100
HSD-A3T	16	13	3	1	33·33
D17S1322	20	12	8	1	12·50
D17S1323	19	5	14	4	28·57

Table 5. Total analysis of LOH in all patients.

Locus	Number of tumours studied	Number of non-informative tumours	Number of informative tumours (x)	Tumours with LOH (y)	Percentage of tumours with LOH (y/x) 100
HSD-A3T	72	47	25	13	52·00
D17S1322	80	35	45	18	40·00
D17S1323	71	12	59	11	18·60

Table 6. Summary of LOH analysis in the three patient categories.

Patient category	Number of tumours studied	Number of non-informative tumours	Number of informative tumours (x)	Tumours with LOH (y)	Percentage of tumours with LOH (y/x) 100
Sporadic cases	40	2	38	23	60·53
Familial cases	20	3	17	7	41·18
Parsi cases	20	4	16	6	37·50
Total analysis	80	9	71	36	50·70

does not assess the risk of breast cancer associated with BRCA1 in any of these three groups. Tumours were shown to have allele loss at BRCA1, confirming its involvement in the molecular pathogenesis of breast cancer and in keeping with its well-documented role of a tumour suppressor gene. Allele loss at BRCA1 is thus an important molecular event in breast tumorigenesis among the Indians and is involved to a high extent of about 50%.

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