ORIGINAL INVESTIGATION

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Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3

Received: 14 October 1994

Abstract Eight Icelandic breast cancer kindreds were subjected to linkage analyses with respect to 28 microsatellite loci dispersed along the short arm of chromosome 3. Breast tumors derived from these kindreds were concurrently scored for allelic imbalance with ten of the markers. Linkage to most markers could be excluded on the basis of negative LOD scores and haplotype analyses, although some moderately positive LOD scores resulted. A high frequency of imbalance in the familial tumors was seen with two of the markers in comparison with results obtained from sporadic material. The highest frequency (68%) of imbalance was detected with the marker D3S1217, which is located on 3p14.2-p14.1. Imbalance at the D3S1211 locus, which is more telomeric (3p24.2-p22), was not significantly elevated in the familial tumors. We suggest that the genetic defect responsible for breast cancer susceptibility in these families either promotes instability in the 3p14.2-p14.1 region or enhances the selective advantage of such changes.

Introduction

Epidemiological evidence suggests that the risk of an individual developing breast cancer is increased significantly when the disease is manifested in close relatives (Claus et al. 1991). The risk for relatives of breast cancer patients is further increased with decreasing age at onset and bilateral disease of the probands. Hence, these two factors serve as indicators of genetic susceptibility. The segregation of breast cancer in high risk families is best explained by assuming an underlying autosomal dominant gene defect and incomplete penetrance (Newman et al. 1988).

Genetic linkage studies have localized a gene predisposing individuals to this disease to a region of chromo-

somes 17q (Hall et al. 1990). This gene, termed BRCA1, is probably responsible for a high fraction of familial breast and ovarian cancers (Easton et al. 1993), and loss of heterozygosity at this site in familial tumors suggests that it is a tumor suppressor gene (Smith et al. 1992). Germline mutations in a strong candidate gene for BRCA1 have been described in affected families (Miki et al. 1994). A second region of linkage in hereditary breast cancer has been localized on chromosome 13q12-13 (Wooster et al. 1994). Breast cancer kindreds showing linkage to this region, which is referred to as BRCA2, are characterized by early onset breast cancer, but they differ from the 17q-linked kindreds in that no excess of ovarian cancer is found. Genetic heterogeneity with respect to 17q and 13q linkage is apparent, and therefore it is likely that genes other than BRCA1 and BRCA2 contribute to familial breast cancer.

Several other genes have been implicated in the onset of breast cancer. Mutations in the P53 gene are responsible for various types of malignancies, including breast cancer, in families affected by the Li-Fraumeni syndrome (Srivastava et al. 1990; Prosser et al. 1992). The androgen receptor is implicated in genetic predisposition to male breast cancer (Wooster et al. 1992). Individuals who are heterozygotes with respect to the ataxia telangiectasia locus have an elevated risk of developing breast cancer, although this locus appears to be unimportant in familial breast cancer (Wooster et al. 1993). Other candidate loci are regions frequently carrying genetic lesions in sporadic breast tumors or sites of genes that are known to be important in breast cancer tumorigenesis. These include regions of frequent loss of heterozygosity in breast tumors; 1q, 3p, 6q, 9q, 11p, 16q, 18q (Devilee et al. 1991; Cornelisse et al. 1992).

Deletions of the short arm of chromosome 3 have been described in human cancers at various sites, including kidney (Kovacs et al. 1988), lung (Hibi et al. 1992), ovary (Ehlen and Dubeau 1990), testis (Lothe et al. 1989), cervix (Yokota et al. 1989), head and neck (Latif et al. 1992), nasopharynx (Huang et al. 1991), and breast (Sato et al. 1991). These findings indicate the presence of one or

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more tumor suppressor genes on 3p. Further support for this is provided by the results of a functional study, in which a small fragment of chromosome 3 (p21-p22) suppressed growth of a highly malignant fibrosarcoma cell line in athymic mice (Killary et al. 1992).

Pandis et al. (1993) have reported an interstitial deletion (del (3)(p12p14)) in four short-term cultures of breast tumors that appear to be clonal. This is the only aberration present in three of the tumors, suggesting that 3p12-14 deletions may be a primary event in the malignant transformation of the breast.

The aim of this study was to evaluate the significance of the short arm of chromosome 3 in familial breast cancer. This was achieved by linkage analyses of eight breast cancer kindreds, by means of 3p microsatellite markers, and by assessment of allelic imbalance in the familial tumors.

Materials and methods

Families

Eight unrelated breast cancer kindreds were included in this study with a total of 64 breast cancer patients (eight bilateral cases, four breast-ovary cases, and one male breast cancer) and 41 individuals diagnosed with cancer at other sites (Table 1). The mean age at onset of breast cancer in the kindreds ranged from 43 to 53 years. One of the eight kindreds in this study (FAM-1) has previously been shown to be linked to the BRCA1 locus (Arason et al. 1993). Cancer in organs other than the breast is noticeable in some of the families, in particular, prostate cancer, which has recently gained attention as a possible risk factor associated with inherited breast cancer susceptibility. Information regarding family members and pedigree structure was obtained from the Icelandic Genealogy Committee, the Icelandic Cancer Registry, pathology records, and death certificates obtained from the Statistical Bureau of Iceland.

Samples

DNA was prepared from whole blood and tumor samples by conventional phenol-extraction methods (Sambrook et al. 1989). Paraffin-embedded tissue of tumor and normal samples was also collected and prepared for polymerase chain reaction (PCR) analysis by the methods described by Smith et al. (1992) and Jackson et al. (1990).

Table 1 Families studied. The number of cancer patients, grouped by site of primary tumor, is given for each kindred

Site of primary tumor	Family number							
	1	2	3	4	5	6	7	8
Breast	4	7	4	5	7	11	3	10
Bilateral breast	2	1	—	2	—	2	1	—
Male breast	—	—	—	—	—	1	—	—
Breast and ovary	1	—	—	1	1	1	—	—
Ovary	—	—	—	—	3	—	—	—
Prostate	1	4	—	4	3	—	2	—
Lung	2	—	2	—	1	2	—	—
Renal	—	—	—	1	—	2	—	—
Colon	2	—	2	—	1	3	—	1
Other sites	2	1	3	6	8	3	—	5

Markers

The 29 markers used in this study are listed in Table 2. They are all localized on 3p, except for D3S1237, which maps to the long arm of the chromosome. The order of marker loci on the chromosome was compiled from the Génethon linkage map (Gyapay et al. 1994), linkage maps available from the GDB (Genome Data Base, The Johns Hopkins University, Baltimore, Maryland, USA) (C3M60), and the GDB physical map assignment. The interval between the 3p markers included in the Génethon linkage map ranged from 8–13 cM, but reached 27 cM in one case (between D3S1263 and D3S1297) near the telomere.

PCR analysis

PCR was carried out in a volume of 30 µl containing 3–10 pmol of each primer, 0.2 mM of each dNTP, 0.5–1 µl (protease-melted) or 50 ng (phenol-extracted) template DNA, 0.3 units thermostable DNA polymerase (Finnzymes Oy), and buffer supplied with the enzyme. One primer in the reaction mixture was end-labeled with γ ATP³² using T4 polynucleotide kinase (Amersham). Samples were processed through 35 cycles comprising 1 min at 94°C, 1 min at the appropriate annealing temperature, and 2 min at 72°C in a Techne PHC-3 thermocycler. Aliquots of the amplified DNA were electrophoresed on standard denaturing 6% polyacrylamide DNA sequencing gels that were exposed to an X-ray film. Standardization within and between gels was achieved by using a mixture of 3 or 4 amplified samples that produced a ladder, preferably containing the smallest and largest bands of the allele spectrum.

Linkage analysis

Two-point linkage analysis was performed using the MLINK program (version 5.03) of the LINKAGE program package (Lathrop and Lalouel 1984). LOD scores were calculated by the assumption of an autosomal dominant disease allele with a frequency of 0.001. Incomplete penetrance was assumed and individuals were assigned to 14 different liability classes on the basis of sex, age, and disease status in accordance with the model constructed for BRCA1 by Easton et al. (1993). Individuals with cancer in organs other than breast were regarded as unaffected in the calculations. Allele frequencies for all marker alleles were initially set as being equal. However, moderately positive LOD scores (> 0.5 , at $\theta = 0$) were recalculated using observed or published allele frequencies, and examination of haplotypes with adjacent markers was also applied whenever a given marker produced LOD scores that exceeded this limit. Linkage analysis in one kindred (FAM-5) is greatly complicated by three apparent consanguinity loops. LOD scores for this family were therefore calculated separately for the two main branches, thereby reducing the number of marriage loops to one. Five breast cancer patients reside in one branch, six in the other and three of these are shared by both branches.

Allelic imbalance

Allelic imbalance in tumors from family members was assessed with eleven markers; D3S1307, D3S1297, D3S1263, D3S656, D3S1211, D3S1289, D3S1029, D3S1285, D3S1217, D3S1284, and D3S1237. Some 35 paired normal and breast tumor samples from seven of the kindreds were available for analysis. The data were compared with the results obtained in a study on sporadic material (Eiríksdóttir et al. 1995) for markers D3S1217, D3S1029, and D3S1211, which were shared in the two studies.

Results

LOD scores for the markers calculated at zero recombination to disease loci are presented in Table 3. The sum of

Table 2 Microsatellite markers used. Distances between markers were obtained from Gyapay et al. (1994). The physical locations and references for each marker are also given

Marker	Distance cM	Cyto. location	Reference
D3S1307		3pter-p25	Weissenbach et al. (1992)
D3S1270	2	3pter-p25	Weissenbach et al. (1992)
D3S1297	27	3pter-p25	Weissenbach et al. (1992)
D3S1263	11	3p25-p24.2	Weissenbach et al. (1992)
D3S1293		3p25-p24.2	Weissenbach et al. (1992)
D3S656	10	3p25.1	Yamakawa et al. (1991c)
D3S1283		3p25-p24.2	Weissenbach et al. (1992)
THRB		3p24.1-p22	Sakurai et al. (1992)
D3S1211	11	3p24.2-p22	Hudson et al. (1992)
D3S1298		3p24.2-p22	Weissenbach et al. (1992)
D3S643		3p21.33-p21.32	Yamakawa et al. (1991c)
D3S966		3p21.32-p21.31	Takahashi et al. (1992)
D3S1029	10	3p21.31-p21.2	Takahashi et al. (1992)
D3S663		3p21.31	Yamakawa et al. (1991b)
D3S1289		3p21.1-p21.1	Weissenbach et al. (1992)
D3S1076		3p21.2-p21.1	Takahashi et al. (1992)
D3S1067	13	3p21.1-p14.3	Yamakawa et al. (1991a)
D3S1312		3p14.2-p14.1	Weissenbach et al. (1992)
D3S1228	9	3p	Jones et al. (1992)
D3S1285		3p14.2-14.1	Weissenbach et al. (1992)
D3S1233		3p14.2-14.1	Naylor (1994, GDB)
D3S1217		3p14.2-14.1	Takahashi et al. (1992)
D3S1210	12	3p14.1-p13	Hudson et al. (1992)
D3S1079		3p13	Takahashi et al. (1992)
D3S659		3p13	Yamakawa et al. (1991c)
D3S1284		3p13-p12	Weissenbach et al. (1992)
D3S1276	8	3p12	Weissenbach et al. (1992)
D3S1101		3p12	Garcia et al. (1991)
D3S1237		3q25.1-q25.2	Weissenbach et al. (1992)

LOD scores for singular markers over all kindreds ranged from -10.664 (D3S1067) to 0.886 (D3S1270). No significant LOD scores were found in any single kindred, and the highest value was found with the marker located on 3q in kindred 2. This region was examined further by applying four additional markers (RHO, D3S196, GLUT2, D3S1232), which all produced negative LOD scores. Application of haplotype analysis also strengthened arguments against 3q linkage in kindred 2.

Haplotype analyses with markers adjacent to those that produced positive results (LOD > 0.5) ruled out the possibility of true linkage of 3p loci with the disease in all the kindreds except families 5B and 7. In family 7, a LOD score of 0.647 was gained with the marker D3S1285. This LOD score was reduced to 0.487 when it was recalculated using allele frequencies obtained from GDB. Haplotypes constructed with the surrounding markers (from D3S1312 to D3S1210) did not exclude linkage. Allelic imbalance is apparent in the tumor material of siblings (nos. 1 and 2: FAM-7; Fig. 1) in this family. If these are assumed to be deletions, then the remaining alleles have a different parental origin.

A moderate LOD score is found in the right branch of family 5 (FAM-5B) in a region spanning D3S1228 to D3S1210. The highest LOD score in this area is given by the marker D3S1217, and this remains unchanged when recalculated with allele frequencies derived from the genotypes of 30 healthy unrelated individuals. The same haplotype is shared in four breast cancer patients, two ovary patients and one breast-ovary cancer patient. In the same branch, two individuals diagnosed with cervical cancer in situ, one with invasive cervical cancer, do not have the haplotype in question. Imbalance is not consistent with linkage, as one of the three tumors (no. 2: FAM-5; Fig. 1) from this kindred, which showed imbalance, had reduced intensity of the founder alleles.

Allelic imbalance with one or more markers on 3p was seen in 26 of the 35 tumors available (see Fig. 1) for analyses. The marker D3S1217 was most frequently involved, as 17 of 25 informative samples showed imbalance at this site. The frequency of imbalance with individual markers progressively increased with proximity to this locus, being lowest near the telomere (D3S1307, D3S1297).

Table 3 LOD scores from two-point linkage analyses; 27 markers on 3p and one marker on 3q (D3S1237) were tested for linkage to breast cancer assuming zero recombination between the marker and disease locus. Family 5 was analyzed as two distinct branches

(FAM-5A, FAM-5B). A sum of the LOD scores for all markers is given with (Total-1) or without (Total-2) inclusion of FAM-1, which shows linkage to the BRCA1 region

Markers	FAM-1	FAM-2	FAM-3	FAM-4	FAM-5A	FAM-5B	FAM-6	FAM-7	FAM-8	Total-1	Total-2
D3S1307	-1.534	-1.274	-0.03	0.168	-0.95	0.004	-2.259	-0.498	0.289	-6.084	-4.55
D3S1270	-0.259	0.797	-0.117	0.199	0.325	-0.117	0.257	-0.498	0.299	0.886	1.145
D3S1297	-1.498	-0.804	-0.017	0.179	-0.914	-0.138	-1.239	0.222	-1.55	-5.759	-4.261
D3S1263	-0.046	-1.691	-0.019	-0.319	0.147	-0.042	-0.977	-0.352	-1.62	-4.919	-4.873
D3S1293	-1.371	-1.691	-0.181	0.702	-0.339	-0.205	-2.132	-0.277	-2.409	-7.903	-6.532
D3S656	-0.854	-2.004	-0.194	-0.478	0.395	-0.181	0.697	0.026	-1.687	-4.28	-3.426
D3S1283	-1.651	-0.084	0.154	-0.787	-0.024	0.145	-2.379	-0.433	-1.831	-6.89	-5.239
THRB	-0.146	-1.262	0.145	0.576	0.002	-0.073	0.503	-0.398	-0.676	-1.329	-1.183
D3S1211	-0.014	-2.334	0.427	-0.707	-1.22	-0.251	-2.476	-0.432	-1.128	-8.135	-8.121
D3S1298	-1.764	-1.442	-0.137	0.921	0.022	-0.098	-0.955	-0.279	-1.647	-5.379	-3.615
D3S643	-3.318	-1.234	-0.139	-1.132	-0.152	-0.157	-2.599	0.065	-0.607	-9.273	-5.955
D3S966	-1.763	-1.383	-0.117	-0.068	0.125	-0.175	-1.838	-0.615	-0.671	-6.505	-4.742
D3S1029	-1.835	0.505	-0.178	0.603	-1.119	-0.283	-1.677	-0.617	-0.624	-5.225	-3.39
D3S663	0	-0.337	-0.139	0.056	-0.17	-0.199	0	0	0	-0.789	-0.789
D3S1289	-3.052	0.515	-0.146	-0.309	-0.603	-0.181	-3.064	-0.286	-1.985	-9.111	-6.059
D3S1076	-2.125	-1.052	0.186	0.354	-0.132	0.141	0.082	-0.493	0	-3.039	-0.914
D3S1067	-3.502	-2.074	-0.17	-1.25	0.031	0.228	-3.188	-0.067	-0.672	-10.664	-7.162
D3S1312	-1.647	-0.002	-0.141	-0.432	-0.603	-0.135	-0.758	0.377	-1.198	-4.539	-2.892
D3S1228	-0.035	-0.635	0.018	-0.622	-0.006	0.16	-1.575	-0.157	0	-2.852	-2.817
D3S1285	-2.481	0.251	0.02	-0.609	-0.343	0.473	-1.959	0.647	0.707	-3.294	-0.813
D3S1233	-2.146	-0.442	-0.106	0.099	-0.005	0.218	-1.729	-0.125	0.418	-3.818	-1.672
D3S1217	-2.524	-0.919	-0.147	0.224	-0.036	0.512	-2.55	0.331	-1.466	-6.575	-4.051
D3S1210	-0.061	-0.748	-0.115	-0.858	-0.375	0.243	-2.915	-0.138	0.208	-4.759	-4.698
D3S1079	-2.419	-0.995	0	0.106	0.037	-0.012	-1.188	0.013	0	-4.458	-2.039
D3S659	-2.548	-1.538	0.132	0.015	-0.387	0.208	-2.336	-0.086	0.067	-6.473	-3.925
D3S1284	-0.391	-0.598	-0.12	-1.221	-1.059	-0.074	-0.133	0.053	-0.146	-3.689	-3.298
D3S1276	0.473	0.385	-0.043	0.082	0.337	0.039	-2.273	-0.024	-1.793	-2.817	-3.29
D3S1101	-0.44	-1.372	-0.107	0.252	-0.725	-0.097	-0.678	0	-1.504	-4.671	-4.231
D3S1237	-3.355	1.109	0.037	-0.179	-0.414	-0.06	-2.24	-0.264	-0.614	-5.98	-2.625

Fig. 1 A chromosomal map of allelic imbalance for the 35 familial breast tumors. Ten markers are located on 3p and one on 3q (D3S1237). The percentage of imbalance for individual markers is given to the right of the map

We compared, by Chi-square analysis, the imbalance in the familial tumors with results obtained from sporadic material for the marker loci D3S1217, D3S1029, and D3S1211 (Table 4). Excess imbalance in the familial tumors was evident with the markers D3S1217 and D3S1029. The number of cases with imbalance in the re-

Table 4 Chi-square test of independence of allelic imbalance with familial cancer involvement (Statistica/Mac Package; Statsoft). The test was performed with and without results for FAM-1 (NS not significant)

Marker	Physical assignment	AI% sporadic	All families		Fam-1 excluded	
			AI% familial	P-value	AI% familial	P-value
D3S1217	3p14.2-p14.1	24 (n = 172)	68 (n = 25)	0.0000	62 (n = 21)	0.0003
D3S1029	3p21.31-p21.2	19 (n = 58)	42 (n = 26)	0.0245	36 (n = 22)	NS
D3S1211	3p24.2-p22	35 (n = 72)	35 (n = 26)	NS	36 (n = 22)	NS

Fig. 2A-D Tumor (T) and normal (N) DNA from patient 1 of family 1 (164) and normal DNA from her mother (166). Imbalance is seen as a diminished signal of the paternal allele in the tumor with the markers D3S1211, D3S1029, and D3S1217. The signal of the maternal allele is decreased when D3S1263 is applied. Arrows Alleles with decreased intensity

gion of D3S1211 was, however, not significantly different between the two studies.

Of the 26 samples, 8 showed imbalance with only one marker, and the remaining 18 had imbalance at two or

more loci. The alleles in cases where 3p imbalance was evident could frequently be given parental assignments. No bias with regard to the origin (parental assignment) of these alleles was detected with any marker. In 14 cases,

the derivation of the alleles was known for at least two loci showing imbalance. In two of these, the alleles with reduced intensity were not derived from the same parental chromosome. In sample 1 (FAM-1), signal reduction of the maternal allele is evident with the marker D3S1263, whereas paternal alleles are reduced in the four remaining loci where imbalance is seen (Fig. 2). This is also evident in sample 2 (FAM-4) where signal reduction of the paternal allele is evident with the marker D3S1217, whereas the maternal signal is reduced in the remaining imbalanced loci (not shown).

Discussion

Strong evidence of genetic linkage to loci on the short arm of chromosome 3 was not found in any of the eight breast cancer kindreds. Two kindreds could not be excluded from linkage on the basis of LOD scores or haplotype analysis. A common region of linkage in these was at the D3S1285 loci. Allelic imbalance in tumors from these kindreds showed that the alleles with retained intensity were not confined to those segregating with the disease, as would be expected if Knudson's two-hit theory for tumor suppressor genes applied. It is unlikely that a germline mutation in a tumor suppressor gene, predisposing to breast cancer, resides at 3p loci in the eight kindreds tested for linkage.

Allelic imbalance of at least one of the 11 marker loci has been detected in 26 of the 35 familial tumors (74%). In comparison with sporadic material, imbalance in familial tumors appears at a significantly higher frequency at the D3S1217 and D3S1029 loci, but not at D3S1211. This is independent of the source of material, as the frequency of imbalance is not statistically different between tumors prepared from fresh tissue or paraffin slices.

Difference in the frequency of allelic imbalance between sporadic and familial tumors appears to be contributed by the D3S1217 locus. Although weak statistical significance is also gained with D3S1029, allelic imbalance at this locus is highly dependent upon AI with D3S1217. As seen in Fig. 1, all the samples with imbalance at either D3S1029 or D3S1289 also display AI at the D3S1217 locus or are uninformative. Further, the frequency of imbalance observed with D3S1029 is not significantly higher in the familial tumors when kindred 1 is disregarded. Imbalance at more telomeric loci, viz., D3S1211, D3S656 and D3S1263, appear with no association of AI at D3S1217, and may consequently represent a second region of 3p instability.

Imbalance on 3p is found in tumors from all kindreds, including the 17q linked kindred (FAM-1). All the tumors that are informative for D3S1217 in kindred 1 have imbalance, and of the two remaining, one shows AI with the adjacent marker D3S1284. Thus, the breast cancer kindreds are homogeneous with respect to instability at 3p14, although they appear to be heterogeneous with respect to 17q linkage and tumor spectrum (Table 1).

Imbalance in 12 of 14 tumors involved signal reduction of alleles from the same parental chromosome. This was also true for the 4 tumors in which the imbalance was

not continual. In two cases, the imbalance affected different parental alleles. These two cases could either reflect a different kind of instability on the same chromosome (amplifications and deletions), or the same type of instability affecting different parental chromosomes.

The observed instability at the 3p14 region in familial tumors can be explained by at least two hypotheses. First, this region could carry a tumor suppressor gene that is more often deleted in the familial tumors, because the sequence of molecular changes leading to malignant growth in familial tumors especially depends on a cooperative effect of a germline mutation and the 3p deletion. Secondly, a germline mutation segregating with the disease in these families may promote genetic instability at specific sites, and the excess of imbalance in familial tumors would therefore be explained by a combination of selection and increased likelihood of the chromosomal change leading to imbalance. In this respect, the physical assignment of D3S1217 to 3p14.2-p14.1 implies proximity to FRA3B, one of the most fragile sites known in the human genome (Wilkie et al. 1994). Chromosomal breaks at this site are induced by aphidicolin, which disturbs the DNA replication process.

The results presented here suggest a distinction between sporadic and familial breast cancer on a molecular basis. Further support of this is offered by the observed elevated frequency of P53 mutations in familial breast tumors (Glebov et al. 1994) and recent findings concerning the BRCA1 gene. Germline mutations in BRCA1 are found in association with a positive history of breast cancer, but somatic changes in the gene have not been described (Futreal et al. 1994). Extended knowledge of the function and specificity of the BRCA1 gene product should help to resolve these problems.

Acknowledgements The authors wish to thank the Nordic primer bank of the Department of Clinical Genetics, University Hospital, Uppsala, Sweden, for providing microsatellite markers. The Nordic primer bank is supported by the Nordic Council of Ministers. We are also grateful to Professor Jonas Hallgrímsson and his staff for providing pathological material and to Ragnheidur Fossdal for help with linkage problems. This work was supported by the Nordic Cancer Union, Icelandic Cancer Society, the Science Fund of Iceland, The University of Iceland Research Fund, the Science Fund of the University Hospital of Iceland, and the Memorial Fund of Bergthóra Magnúsdóttir and Jakob B. Bjarnason.

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