Chromosome 8p alterations in sporadic and *BRCA2* 999del5 linked breast cancer

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Abstract

Chromosomal losses involving the short arm of chromosome 8 are frequent in a variety of tumour types, including breast cancer, suggesting the presence of one or more tumour suppressor genes in this region. In this study, we have used 11 microsatellite markers to analyse loss of heterozygosity (LOH) at chromosome 8p in 151 sporadic breast tumours and 50 tumours from subjects carrying the *BRCA2* 999del5 mutation. Fifty percent of sporadic tumours compared to 78% of *BRCA2* linked tumours exhibit LOH at one or more markers at 8p showing that chromosome 8p alterations in breast tumours from *BRCA2* 999del5 carriers are more pronounced than in sporadic breast tumours. The pattern of LOH is different in the two groups and a higher proportion of *BRCA2* tumours have LOH in a large region of chromosome 8p. In the total patient material, LOH of 8p is associated with LOH at other chromosome regions, for example, 1p, 3p, 6q, 7q, 9p, 11p, 13q, 17p, and 20q, but no association is found between LOH at 8p and chromosome regions 11q, 16q, 17q, and 18q. Furthermore, an association is detected between LOH at 8p and positive node status, large tumour size, aneuploidy, and high S phase fraction. Breast cancer patients with LOH at chromosome 8p have a worse prognosis than patients without this defect. Multivariate analysis suggests that LOH at 8p is an independent prognostic factor. We conclude that chromosome 8p carries a tumour suppressor gene or genes, the loss of which results in growth advantage of breast tumour cells, especially in carriers of the *BRCA2* 999del5 mutation.

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Keywords: chromosome 8; *BRCA2*; LOH; breast cancer

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Breast cancer is one of the most common malignancies in women living in western countries, and approximately 5-10% of the cases are thought to be the result of a hereditary predisposition to the disease.[1] Germline mutations in *BRCA2* confer an increased risk for both female and male breast cancer.[2 3] Other malignancies, such as cancer of the prostate, ovary, pancreas, larynx, cervix, and ureter and malignant melanoma of the eye, are also seen more frequently in *BRCA2* mutation carriers.[4] Women who carry germline mutations in the *BRCA1* or *BRCA2* genes tend to develop breast cancer at an early age as well as being at increased risk of bilateral breast cancer. Somatic loss of the wild type allele in tumours of *BRCA1* and *BRCA2* mutation carriers suggests that these genes function as tumour suppressor genes.[5–7] In the breast, *BRCA1* and *BRCA2* mRNA expression are induced during puberty and pregnancy, suggesting a regulation by sex hormones.[8]

More frequent alterations are detected in the genome of tumours from *BRCA2* carriers than sporadic tumours, suggesting a specific or more aggressive tumour progression pathway of breast cancer in the presence of a germline mutation.[9 10] Failure of the DNA repair mechanism owing to dysfunctional Brca2 protein could be responsible for this instability. Chromosome alterations in male breast tumours of *BRCA2* mutation carriers are similar to those identified in the corresponding *BRCA2* associated female breast cancer, suggesting that despite hormonal differences between females and males, similar genetic changes occur.[11] As in human breast tumours with dysfunctional Brca2 protein, cells from *BRCA2* -/- knockout mice show accumulation of chromosome abnormalities.[12] A human pancreatic adenocarcinoma cell line lacking functional copies of the *BRCA2* gene is defective in repairing double strand DNA breaks induced by ionising radiation or drugs.[13] This suggests that Brca2 defective cancer cells are highly sensitive to agents that cause double strand breaks in DNA.

The search for new breast cancer susceptibility genes is currently intense and involves various procedures. Loss of heterozygosity (LOH) is one of the most frequent alterations in solid tumours and the identification of areas with a high LOH will point to the characterisation of chromosomal regions harbouring putative tumour suppressor genes. LOH at chromosome 8p has been found to occur in a number of types of human cancer, including those of the colon and rectum,[14–16] bladder,[17] liver,[18 19] prostate,[20 21] lung,[19–22] ovary,[19–23] and breast.[24–26] Recent studies suggest that this chromosome arm has one or more tumour suppressor genes frequently involved in breast cancer.[24–28] Linkage analyses of French and German breast cancer families have provided evidence for a familial breast cancer susceptibility gene on chromosome arm 8p12-p22.[24 28]

Attempts have been made to determine when LOH of chromosomal arm 8p occurs in breast cancer development. Anbazhagan *et al*[26] reported LOH at 8p to be common in ductal carcinoma in situ (DCIS) suggesting that 8p LOH might be a relatively early event in breast

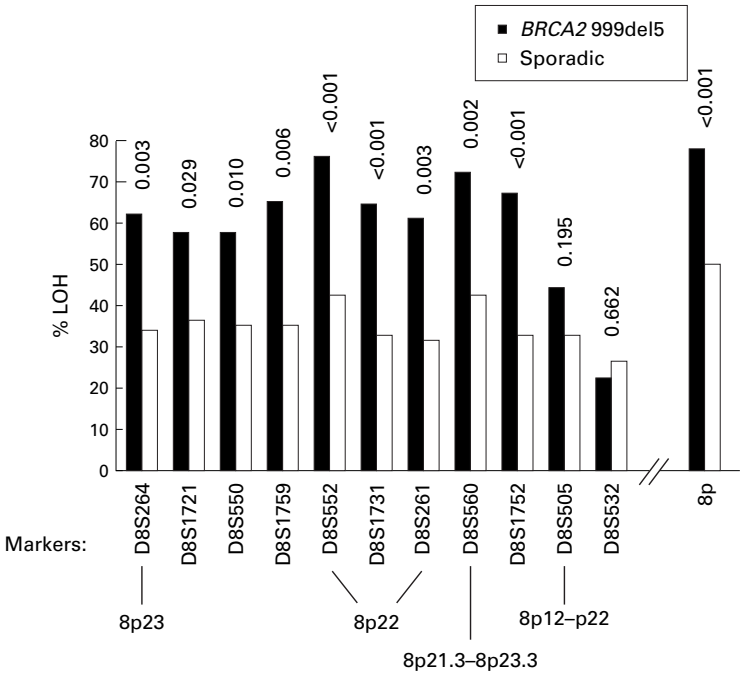

Figure 1 Comparison of LOH at 8p in BRCA2 tumours and sporadic tumours. P values determined by chi-square tests comparing the frequency of LOH in the two patient groups are shown at the top of the bars. The bars to the right (8p) represent the frequency of LOH with at least one marker in the two patient groups.

carcinogenesis. Radford *et al*[29] found allelic loss at 8p in 19% of DCIS suggesting inactivation of tumour suppressor genes at 8p to be an early event in the tumorigenesis of breast cancer. Yaremko *et al*[30] suggested that 8p LOH plays a role in breast carcinogenesis at the point where tumours progress from non-invasive to infiltrating ductal carcinomas.

BRCA2 999del5 germline mutation has been found in 8% of patients diagnosed with breast cancer in Iceland.[31 32] In this study, 50 tumours from breast cancer patients with *BRCA2* 999del5 germline mutation and 151 sporadic breast cancers were tested for LOH using 11 microsatellite markers mapping to chromosome 8p. Furthermore, we compared tumours with and without LOH at 8p with respect to clinicopathological features to determine whether LOH at 8p is of prognostic value for breast cancer patients. We also compared LOH at 8p with LOH at other chromosomal arms in an attempt to determine a common pathway for genetic events in breast tumours.

Materials and methods

PATIENTS AND TUMOUR MATERIAL

In all, 201 fresh biopsies from primary breast tumours were analysed, 50 tumours from patients with *BRCA2* 999del5 germline mutation and 151 sporadic tumours. Blood samples from the patients were collected in EDTA and, if not processed immediately, tumours and blood were quickly frozen at −70°C. The blood samples were screened for the *BRCA2* 999del5 mutation. All information about the tumours, for example, size, type, and node status, was recorded by the Department of Pathology, National Hospital of Iceland. The average age at diagnosis of the patients with *BRCA2* 999del5 mutation was 49 years and 60 years in the sporadic patients.

DNA EXTRACTION AND ANALYSIS

Standard procedures were used for extracting tumour DNA from the nuclear pellet remaining after cytosol removal for the hormone receptor analysis or pulverised primary tumour tissue.[33] Normal DNA was extracted from peripheral blood leucocytes and matched with tumour DNA.[34] The DNA was subjected to PCR amplification using DynaZyme Polymerase (Finnzymes Oy, Espoo, Finland) in the buffer solution provided by the manufacturer. The PCR amplification was carried out in 25 µl reaction volumes in 96 well plates (Techne), using 30 ng of genomic DNA, 5 pmol of the forward and reverse primers, 2.5 nmol of each dNTP, and 0.5 units DynaZyme polymerase. The samples were subjected to 35 cycles of

Table 1 The markers used in this study, frequency of heterozygosity, and LOH in sporadic tumours and tumours from subjects carrying the BRCA2 999del5 mutation

			Sporadic samples			*BRCA2 samples*		
Marker	*Distance (cM*)*	*Location*	*No of samples tested*	*Informative samples (%)*	*Tumours with LOH (%)*	*No of samples tested*	*Informative samples (%)*	*Tumours with LOH (%)*
D8S264		8p23	141	114 (81)	39 (34)	48	37 (77)	23 (62)
	14.2							
D8S1721			143	100 (70)	36 (36)	47	37 (79)	21 (57)
	5.5							
D8S550			143	124 (87)	43 (35)	48	44 (92)	25 (57)
	0.5							
D8S1759			144	81 (56)	28 (35)	48	26 (54)	17 (65)
	4.9							
D8S552		8p22	145	118 (81)	49 (42)	50	33 (66)	25 (76)
	4.9							
D8S1731			144	116 (81)	37 (32)	48	45 (94)	29 (64)
	5.1							
D8S261		8p22	145	102 (70)	32 (31)	47	33 (70)	20 (61)
	6.4							
D8S560		8p21.3-p23.3	142	99 (70)	42 (42)	48	36 (75)	26 (72)
	2.7							
D8S1752			142	117 (82)	37 (32)	49	39 (80)	26 (67)
	15.1							
D8S505		8p12-p22	147	115 (78)	37 (32)	48	39 (81)	17 (44)
	4.6							
D8S532			148	124 (84)	32 (26)	48	36 (75)	8 (22)

*Markers ordered according to the genetic map provided by the Genome Data Base (GDB).

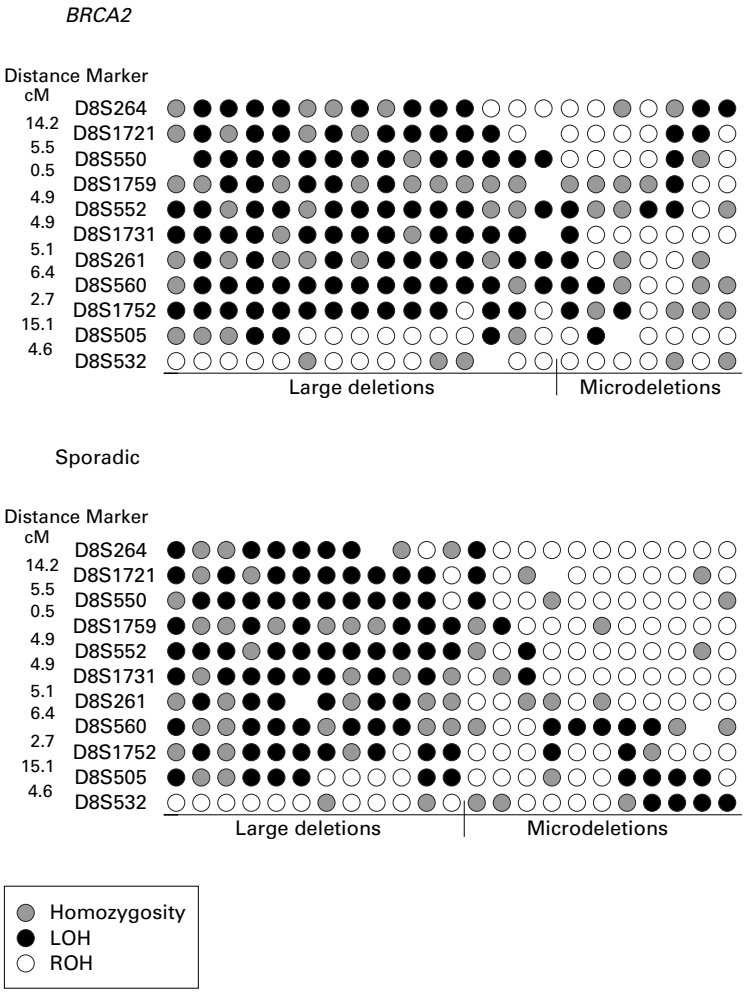

Figure 2 Pattern of deletions in chromosome 8p in selected tumours with large deletions and microdeletions.

amplification, consisting of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C, followed by final extension for 10 minutes at 72°C. A hot start was used by adding the enzyme during the first cycle at about 72°C, after a preincubation time of five minutes at 94°C. Distances between the markers were obtained from Gyapay *et al*.[35] The microsatellite markers used (D8S264, D8S1721, D8S550, D8S1759, D8S552, D8S1731, D8S261, D8S560, D8S1752, D8S505, and D8S532) were obtained from Research Genetics (Huntsville, Alabama, USA).

The PCR products were subjected to denaturing gel electrophoresis in 6.5% polyacrylamide, 8 mol/l urea denaturing gel, transferred to a positively charged nylon membrane, Hybond-N$^+$ (Amersham, Aylesbury, UK), and heated for at least two hours at 80°C or fixed by exposing it to UV light for one minute 40 seconds. The non-radioactive detection method to visualise the PCR products is based on a method developed by Vignal *et al*[36] with minor changes that have been previously described.[37] Autoradiographs were inspected visually by at least two reviewers, comparing the intensity of alleles from normal and tumour DNA. Absence or significant decrease (more than 50%) in the intensity of one allele in the tumour compared to the normal reference sample was considered as LOH. S phase fraction and DNA ploidy were measured using a FACScan flow cytometer (Becton-Dickinson, San José, California, USA). Oestrogen and progesterone receptors (ER and PgR) were analysed by using a ligand binding assay.

STATISTICAL ANALYSIS

A chi-square test was used to assess the relationship between LOH at 8p and clinicopathological variables. Survival curves were calculated according to the method of Kaplan and Meier.[38] Tests of difference between survival curves were made with the log rank test for censored survival data.[39] Multivariate analysis was performed with Cox´s partially non-parametric regression model.[40] The Survival Tools for Statview package (Abacus Concepts Inc, Berkeley, CA) were used for the statistical analyses.

Results

Fifty breast tumours from patients with *BRCA2* 999del5 germline mutation and 151 sporadic breast tumours were tested for loss of heterozygosity (LOH) using 11 markers mapping to chromosome 8p. The results are shown in table 1 and fig 1. In general, tumours from *BRCA2* 999del5 showed a higher frequency of LOH (22-76%) than sporadic tumours (26-42%), as shown in fig 1, but the pattern of LOH was similar in both groups. A significant difference was detected with all markers except D8S505 and D8S532. In all, 39 of the 50 tumours from *BRCA2* carriers (78%) showed LOH with at least one marker compared to 75 of the 151 sporadic tumours (50%). Two LOH peaks were detected at markers D8S552 (8p22) and D8S560 (8p21.3-p23.3) in both groups.

The pattern of LOH is shown in fig 2. Large deletions between markers D8S550 and D8S1752 were detected in 17% of sporadic tumours with LOH, compared to 42% of *BRCA2* tumours, with a significant difference between the groups (p=0.004). Fifteen percent of sporadic tumours with LOH at 8p and 16% of *BRCA2* tumours with LOH at 8p showed microdeletions. Thirty eight percent of sporadic tumours and 13% of *BRCA2* tumours with LOH at 8p showed a complex LOH pattern (data not shown), which is a significant difference (p=0.007). Thirty percent of sporadic tumours and 29% of *BRCA2* tumours with LOH at 8p showed LOH with all informative markers (data not shown). *BRCA2* tumours show microdeletions between markers D8S550 and D8S1731 and between markers D8S261 and D8S532. The sporadic tumours show microdeletions between markers D8S1759 and D8S552 and between markers D8S261 and D8S1752 (fig 2).

Owing to the small number of tumours analysed, both patient groups were combined when comparing tumours with and without LOH at 8p with clinicopathological features, deletions at other chromosomal arms, and survival. Table 2 shows the chi-square analysis

Table 2 Loss of heterozygosity (LOH) at chromosome 8p compared with LOH at other chromosome regions

Chromosome A	*LOH 8p/ROH A*	*LOH%*	*LOH 8p/LOH A*	*LOH%*	*p value*
1p	22/46	48	67/103	65	0.048*
3p	36/86	42	66/87	76	<0.0001***
6q	23/64	36	50/68	74	<0.0001***
7q	38/92	41	34/50	68	0.002**
9p	34/81	42	38/49	78	<0.0001***
11p	25/46	54	24/26	92	0.001**
11q	26/50	52	44/73	60	0.36
13q	11/40	28	53/69	77	<0.0001***
16q	25/55	45	54/93	58	0.137
17p	14/31	45	15/19	79	0.037*
17q	18/33	55	43/59	73	0.074
18q	23/51	45	68/113	60	0.072
20q	52/114	46	49/63	78	<0.0001***

The first column shows the other 13 chromosome arms (A) analysed. The second and third columns show the fraction and percentage of tumours with 8p LOH in total tumours with ROH of chromosome A. The fourth and fifth columns show the fraction and percentage of tumours with 8p LOH in total tumours with LOH at the given chromosome A. The calculated p values are in the last column (chi-square).
ROH: retention of heterozygosity.
*95% confidence interval. **99% confidence interval. ***99.9% confidence interval.

comparing LOH at 8p with LOH at other chromosome regions investigated in our laboratory. There was a significant association between LOH at 8p and LOH at the following chromosomal regions: 1p, 3p, 6q, 7q, 9p, 11p, 13q, 17p, and 20q. The highest frequency of LOH at 8p in breast tumours with LOH at another chromosome region was in tumours with LOH at 11p. Of 26 tumours showing LOH at 11p, 24 also showed LOH at 8p (92%). Similarly, of 23 tumours with ROH (retention of heterozygosity) at 8p, only two showed LOH at chromosome 11p (9%). There was no association between LOH at 8p and LOH at 11q, 16q, 17q, and 18q.

Results from the chi-square analysis comparing LOH at 8p with clinicopathological variables are shown in table 3. There was a significant association between LOH at 8p and positive node status (p=0.033), LOH at 8p and tumour size (p=0.035), LOH at 8p and DNA ploidy (p=0.002), and between LOH at 8p and high S phase fraction (p=0.004). There was no significant association between LOH at 8p and parameters such as histological type of the tumour, ER content, PgR content, or age at diagnosis.

Survival analyses with a median follow up time of five years showed a significant difference between survival curves in breast cancer patients with tumours with LOH at 8p and the patients without LOH at 8p (p=0.012). Fig 3 shows a graphic presentation of the survival statistics for the patient groups with and without LOH at 8p. In a multivariate analysis using Cox's regression methods, positive lymph nodes, LOH at 8p, and low ER content were shown to be of prognostic value. Patients with tumours with LOH at 8p had a 1.7 fold increase in relative mortality rate compared with patients without LOH at 8p. The 95% confidence interval was 1.0-2.9 and the p value was 0.039 (table 4). Neither S phase fraction nor tumour size proved to be an independent prognostic factor in this patient collection.

Table 3 LOH at 8p (with at least one marker) in breast tumour DNA compared with clinical and pathological parameters by chi-square analysis

Variable	*LOH/total*	*%*	*p value*
All samples	114/201	57	
Node status			
Negative	51/106	48	
Positive	54/85	64	0.033*
No information	9/10		
Tumour size			
<2 cm	39/84	46	
≥2 cm	66/107	62	0.035*
No information	9/10		
Histological type			
Ductal	101/178	57	
Lobular	5/13	38	0.20
No information	8/10		
ER fmol/mg protein			
≥10	76/140	54	
<10	32/53	60	0.45
No information	6/8		
PgR fmol/mg protein			
≥25	55/104	53	
<25	51/86	59	0.38
No information	8/11		
Ploidy			
Diploid	26/64	41	
Aneuploid	66/102	65	0.002**
No information	22/35		
S phase			
Low (<7%)	35/81	43	
High (≥7%)	49/74	66	0.004**
No information	30/46		
Age at diagnosis			
<50 years	42/71	59	
≥50 years	65/123	53	0.39
No information	7/7		

*95% confidence limit.
**99% confidence limit.

Discussion

The results presented here indicate that LOH in the 8p region is more frequent in carriers of the 999del5 mutation than in a control group without *BRCA2* germline mutation. The *BRCA2* 999del5 tumours also show that larger regions are involved compared to sporadic tumours and there is a difference in the pattern of microdeletions in the two groups as the microdeletions are larger in *BRCA2* linked tumours. These results suggest a more aggressive tumour progression pathway in patients predisposed to breast cancer resulting from *BRCA2* germline mutation. Our findings may pinpoint candidate loci in the search for genes that when inactivated promote tumour progression in people predisposed to breast cancer resulting from the *BRCA2* 999del5 mutation.

In sporadic and *BRCA2* tumours the highest frequency of LOH is detected with markers D8S552 and D8S560 at chromosome 8p21.3-p22 and significantly higher in the *BRCA2* tumours. Microdeletions are also detected in both groups in the same region. In familial breast cancer negative for *BRCA1* and *BRCA2* mutations, a positive lod score was obtained with markers D8S133, NEFL, and D8S259, located between markers D8S1731 and D8S261, D8S1752 and D8S505, and D8S505 and D8S532, used in this study, respectively.[24 41] In tumours of family members, the same chromosome region shows high LOH.[24 41] Furthermore, this chromosome region has been reported with increased LOH in sporadic female and male breast cancer that is associated with cancer progression.[25–27 30] Therefore, we conclude that the 8p12-p22 chromosome region that we report here with high LOH and microdeletions, especially in tumours from subjects carrying the *BRCA2* 999del5 mutation, is consistent with the

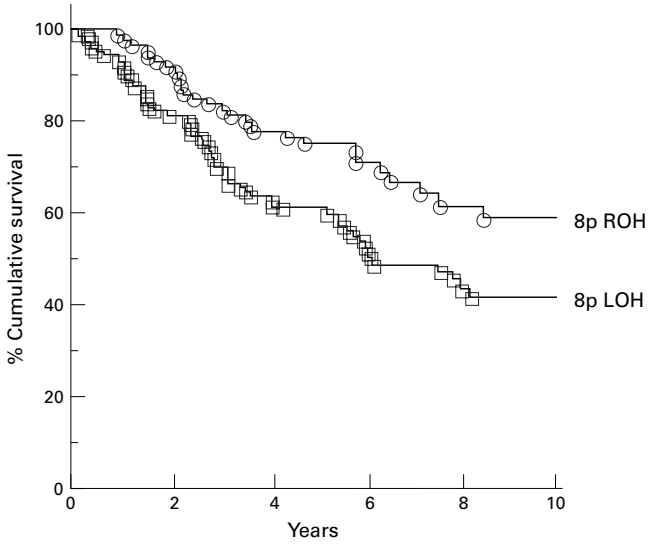

Figure 3 Cumulative percentage of survival at different time intervals. The median follow up time is five years and the p value is 0.012. The numbers of patients at risk with and without LOH at 8p at 0, 3, 6, and 9 years are shown.

*Table 4 Multivariate analysis of survival in patients with breast cancer**

	All patients (n=201)		
Factor	*Univariate p value*	*Multivariate p value*	*RR (95% CI)*
Axillary node involvement	0.002	0.015	1.9 (1.1–3.1)
LOH at 8p	0.012	0.039	1.7 (1.0–2.9)
S phase fraction	0.003	0.173	1.4 (0.9–2.4)
Tumour size	0.004	0.770	1.1 (0.6–1.9)
ER	0.002	0.004	2.2 (1.3–3.8)

*Proportional hazard (Cox) regression. Factors were categorised as shown in table 2. RR, relative risk of dying.

location of 8p LOH in previous studies and the region of published breast cancer linkage of 8p.

A difference in pathogenesis in tumours from subjects carrying *BRCA2* germline mutations and sporadic tumours has been described.[42] Several chromosome regions show more frequent alterations in *BRCA2* tumours than in sporadic tumours. Ingvarsson *et al*[10] found that the frequency of LOH was similar in some chromosomal regions in the *BRCA2* 999del5 and sporadic tumours but significantly different in others. Tirkkonen *et al*[11] used comparative genomic hybridisation to evaluate the difference in chromosome alterations between sporadic tumours and tumours from subjects with germline mutation in *BRCA1* or *BRCA2* genes and found that some regions were more frequently altered in *BRCA1* or *BRCA2* linked tumours than in the control group, while other regions did not show a significant difference between the groups. Changes seen preferentially in *BRCA2* linked tumours may pinpoint regions where tumour suppressor genes might be located.[9] This probably reflects the role of Brca2 protein in DNA repair and maintaining the integrity of the genome, as has been suggested by knockout mice experiments.[12 43]

High LOH at the *TP53* locus, overexpression of the p53 protein, and increased somatic mutation of the *TP53* gene have been found in tumours from *BRCA2* mutation carriers.[44 45] LOH and expression studies of the *FHIT* gene at 3p14.2 and Fhit protein in *BRCA2* linked and sporadic cancer have shown its loss in a significant fraction of sporadic breast cancers and in a larger fraction of breast cancers from subjects with an inherited *BRCA2* mutation.[46 47] The common fragile site FRA3B is located in the *FHIT* gene, presumably explaining the increased frequency of LOH in *BRCA2* linked tumours owing to defective repair function. A majority of *BRCA2* mutated tumours also show LOH at 6q, 11p, 11q, and 13q, each showing three common fragile sites. Conversely, 16q exhibits two common fragile sites near a region of loss in sporadic breast cancer, but 16q was not increased in the *BRCA2* mutant carriers and 17p does not show a common fragile site, but increased LOH is observed in *BRCA2* linked tumours.[10] Similarly, 8p does not exhibit a fragile site although it has a high LOH in *BRCA2* linked tumours compared to sporadic tumours. Thus, presence of a common fragile site alone cannot explain increased LOH frequencies in *BRCA2* linked tumours.

LOH at 8p was compared with previous deletion studies done in our laboratory on the same tumour material, and we found a significant association between LOH at 8p and 3p, 6q, 7q, 9p, 11p, 13q, and 20q, and a weaker association between LOH at 8p and 1p and 17p. The results suggest a putative tumour suppressor gene in the region tested that, in combination with other deletions, may enhance tumour growth.

LOH at 8p was compared with various prognostic variables. No association was found with histological type, ER/PgR content, or age at diagnosis. LOH at 8p was associated with aneuploidy and high S phase fraction, suggesting that loss of a gene on 8p can affect the control of cell proliferation and the maintenance of genome stability. There was also a weak association between LOH at 8p and tumour size and node status, suggesting that LOH at 8p enhances tumour growth and nodal metastasis progression in carcinogenesis. Survival analyses showed that patients with tumours where LOH at 8p was detected have a significantly worse prognosis than patients where LOH is not observed. Deletions in this region were of independent prognostic value. These findings suggest that information on LOH at 8p could be useful as a prognostic factor.

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