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Original Paper

Mapping Loss of Heterozygosity at Chromosome 13q: Loss at 13q12-q13 is Associated with Breast Tumour Progression and Poor Prognosis

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Several chromosome regions exhibit loss of heterozygosity (LOH) in human breast carcinoma and are thought to harbour tumour suppressor genes (TSG). At chromosome 13q, two TSGs have been identified, *RB1* at 13q14 and *BRCA2* at 13q12-q13. In this study, 139 sporadic breast tumours were analysed with 18 polymorphic microsatellite markers for detailed mapping of LOH at chromosome 13q and evaluation of an association with known progression factors. LOH with at least one marker was observed in 71 (51%) of the tumours analysed. The deletion mapping indicated three LOH target regions, 13q12-q13, 13q14 and 13q31-q34. LOH at chromosome 13q12-q13 was associated with low progesterone receptor content, a high S phase fraction and aneuploidy. Multivariate analysis adjusting for lymph node involvement and S phase fraction showed that patients with tumours exhibiting LOH at 13q12-q13 have a 3–4-fold increased risk of recurrence and death compared with other patients. Our results suggest there are at least three separate LOH target regions at chromosome 13q and inactivation of one or more genes at chromosome 13q12-q13 results in poor prognosis for breast cancer patients.

Key words: breast cancer, deletion mapping, chromosome 13q, *BRCA2*, *RB1*, prognosis
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INTRODUCTION

PROGRESSION OF human solid tumours is the result of an accumulation of multiple genetic alterations that gradually lead to a fully malignant phenotype [1, 2]. Cytogenetic and molecular analyses of breast cancer have revealed numerous chromosomal abnormalities that might be involved in breast cancer development. The chromosome regions most frequently involved include those on 1p, 1q, 3p, 6q, 8p, 11p, 11q, 13q, 16q, 17p, 17q, 18q and 20q [3, 4]. At some of these chromosome regions, genes have been identified that are found to be mutated in breast tumour cells. These include the tumour suppressor genes (TSGs) *TP53* and *RB1* and the oncogenes *ERBB2*, *MYC* and *CCNDI*. Apart from increasing the understanding of the biology of breast cancer, the ultimate aim of these studies was to provide markers for early diagnosis of cancer, prognosis and selection of treatment. Studies have shown that amplification of *ERBB2* in breast cancer correlates with increased relapse and poorer survival [5]. Patients with tumours with *TP53* mutations have been shown to be associated with early relapse and patients who are also lymph node positive are less responsive to adjuvant therapy than patients without *TP53* mutations [6]. However, the putative gene targets at most of the chromosome regions found to be affected in breast tumours are still unknown.

Regions on chromosome 13q are frequently lost in primary tumours in various tissues, i.e. pituitary, liver, prostate, pancreas and ovary [7–11]. Several studies have also detected loss in breast tumours at a frequency varying from 25–63% [12–17]. Two TSGs have been identified at chromosome 13q. These are the retinoblastoma gene (*RB1*) located at 13q14 [18] and the breast cancer susceptibility gene

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(*BRCA2*) at 13q12-q13 [19], found to be mutated in more than 30% of high risk breast cancer families. Deletion studies on chromosome 13q have mainly focused on the chromosomal regions of these genes. Loss of heterozygosity (LOH) at *RB1* has been detected in breast tumours, indicating that the inactivation of the *RB1* gene may play a role in human breast carcinogenesis [17, 20, 21]. However, deletions at the *RB1* locus in breast cancer are not correlated with loss of pRb protein expression [22], suggesting the presence of another TSG adjacent to *RB1*. Studies on cell lines indicate that the chromosomal region including the *BRCA2* gene is also a distinct target for LOH on chromosome 13 in addition to *RB1* [12]. It has been suggested that loss of function of the *BRCA2* gene or another gene located close to it, might also be important for sporadic tumour development [17, 23]. Mutations in *BRCA2* seem to be rare in sporadic tumours [24], but additional means of inactivation of *BRCA2* have not been excluded.

The aim of the present study was the detailed mapping of LOH at chromosome 13q in sporadic breast tumours and the determination of the prognostic value of the deletions. We analysed 139 sporadic breast tumours using 18 microsatellite markers spanning the entire chromosomal arm. The results suggest that there are at least three LOH target regions at chromosome 13q and that LOH at chromosome 13q12-q13 is associated with a high rate of cell proliferation and poor prognosis of the patients.

MATERIALS AND METHODS

Patients and tumour material

A fresh biopsy from primary breast tumours is routinely sent to our laboratory for oestrogen and progesterone receptor (ER and PgR) analysis. Blood samples from the patients are collected in ethylene diamine tetra acetic acid (EDTA) and, if not processed immediately, the tumours and blood are quick frozen at -70°C. Tumours were chosen randomly from the material sent to our laboratory and screened for the 999del5 *BRCA2* mutation found to be involved in the majority of hereditary breast cancer families in Iceland [25, 26]. The mutation is found in 8.5% of Icelandic breast cancer patients [27]. Excluding samples from patients carrying the 999del5 mutation, 139 patients proved informative in this study. The average age at diagnosis ranged from 30 to 95 years (mean 60 years), 65 patients were positive for one or more lymph node and the median follow-up time was 3.4 years.

DNA extraction and analysis

A salting out procedure [28] and phenol extraction methods were used to obtain DNA from whole blood and tumour samples, respectively. Paired blood and tumour DNA was subjected to polymerase chain reaction (PCR) analysis using DynaZyme™ polymerase (Finnzymes Oy, Espoo, Finland) in the buffer solution provided by the manufacturer. The markers used were obtained from Research Genetics (Huntsville, Alabama, U.S.A.) and Pharmacia Biotech (Hørsholm, Denmark). Eighteen markers were applied spanning 13q: *D13S217*, *D13S1246*, *D13S260*, *D13S171*, *D13S1695*, *D13S1694*, *D13S267*, *D13S219*, *D13S263*, *D13S155*, *D13S153*, *D13S176*, *D13S276*, *D13S160*, *D13S154*, *D13S158*, *D13S173* and *D13S285*. Marker *D13S153* is located within the *RB1* gene [29]. The distances between the markers were obtained from Gyapay and colleagues [30] and Couch and associates [31]. According to Wooster and colleagues [32], the *BRCA2* gene lies in the 1 cM region between markers *D13S260* and *D13S171*. The non-radioactive method used for DNA detection is based on a method developed by Vignal and associates [33], with minor changes that have been previously described [34]. Autoradiograms were inspected visually by two viewers, comparing the intensity of alleles from normal and tumour DNA. Any absence or significant decrease of one allele relative to the other was considered LOH. Due to normal cell contamination in the tumour samples, a conservative estimate of LOH was obtained. S phase fraction and aneuploidy was measured using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, California, U.S.A.).

Statistical analysis

A chi-square test was used to assess the relationship between clinical–pathological variables and LOH at one or more markers at 13q. The variables were categorised using commonly applied cut-off levels. Survival curves were calculated according to the method of Kaplan and Meier. Overall survival was calculated from the date of surgery to death or the date patients were last known to be alive. Disease-free survival was calculated from the date of surgery until relapse or the date patients were last known to be disease free. Differences between curves were estimated with the log-rank test for censored survival data. Multivariate analyses were performed with Cox's partially non-parametric regression model. Survival Tools for StatView (Abacus Concepts, Berkeley, California, U.S.A.) was used for the statistical analyses.

The statistical analyses were repeated for the LOH results at three separate LOH target regions; 13q12-q13, 13q14 (the *RB1* region) and 13q31-q34. The analyses were obtained by grouping markers mapping to the specific regions. Chromosome region 13q12-q13 was represented by markers *D13S260*, *D13S171*, *D13S1695*, *D13S1694* and *D13S267*. Chromosome region 13q14 was represented by the intragenic marker for *RB1* (*D13S153*). Chromosome region 13q31-q34 included markers *D13S160*, *D13S154*, *D13S158*, *D13S173* and *D13S285*.

RESULTS

Deletion analysis

Sporadic breast tumours were screened for LOH using 12 microsatellite markers at approximately 10 cM intervals on chromosome 13q. More markers were added for further deletion mapping near the known TSGs, making a total of 18 markers (Table 1). A total of 139 cases were analysed. The frequency of deletions observed with the markers ranged from 18 to 42% (Table 1). Two major peaks of LOH were observed, one at the 13q12-q14 region and the other at the 13q31-q34 region. LOH with at least one of the markers was observed in 71 (51%) of the tumours. Figure 1 shows 46 tumours with selective LOH. The 15 tumours that showed total loss of the chromosome and 10 tumours that had no information regarding chromosome region 13q31-q34 are not included in the deletion map. Figure 1 is also representative of the distribution of the number of markers showing LOH per tumour. The results indicate that three regions are possible targets for LOH in the breast tumours studied here: (i) 13q12-q13 (groups A, B, and C); (ii) 13q14 harbouring *RB1* (group C); and (iii) the telomeric region 13q31-q34 (groups B and C).

Thirty-one tumours (28 shown in Figure 1), were observed with LOH at 13q12-q13 and retention of heterozygosity at

Table 1. *Results of the loss of heterozygosity (LOH) analysis at chromosome* 13q; 139 *breast tumours were analysed. The table shows the number of samples analysed, the number of informative tumours (heterozygotes) and the percentage LOH detected*

Marker	Distance (cM)	Cytological location	Number of samples	Number of heterozygotes	LOH (%)	
D13S217		13q12	111	79	29	
	8					
D13S1246		13q12	129	103	34	
	3					
D13S260		13q12.3	135	106	35	
	1					*BRCA2*
D13S171		13q12.3-q13	132	95	40	
D13S1695		13q12-q13	104	79	42	
	2					
D13S1694		13q12-q13	116	75	40	
D13S267		13q12.3	133	105	42	
	2					
D13S219		13q12.3-q13	114	68	40	
	10					
D13S263		13q14.1-q14.2	105	81	30	
	8					
D13S155		13q14.3-q21.2	106	85	25	
	2					
D13S153		13q14.1-q14.3	130	115	21	*RB1*
	4					
D13S176		13q14.3	113	85	18	
	4					
D13S276		13q14.3-q21.1	108	80	24	
	10					
D13S160		13q21.1-q31	111	86	22	
	15					
D13S154		13q31-q32	111	100	24	
	11					
D13S158		13q31-q32	108	84	29	
	11					
D13S173		13q32-q34	112	89	27	
	18					
D13S285		13q34	113	100	25	

Cytological information and distances between markers was obtained from the Genome Data Base, Baltimore, U.S.A..

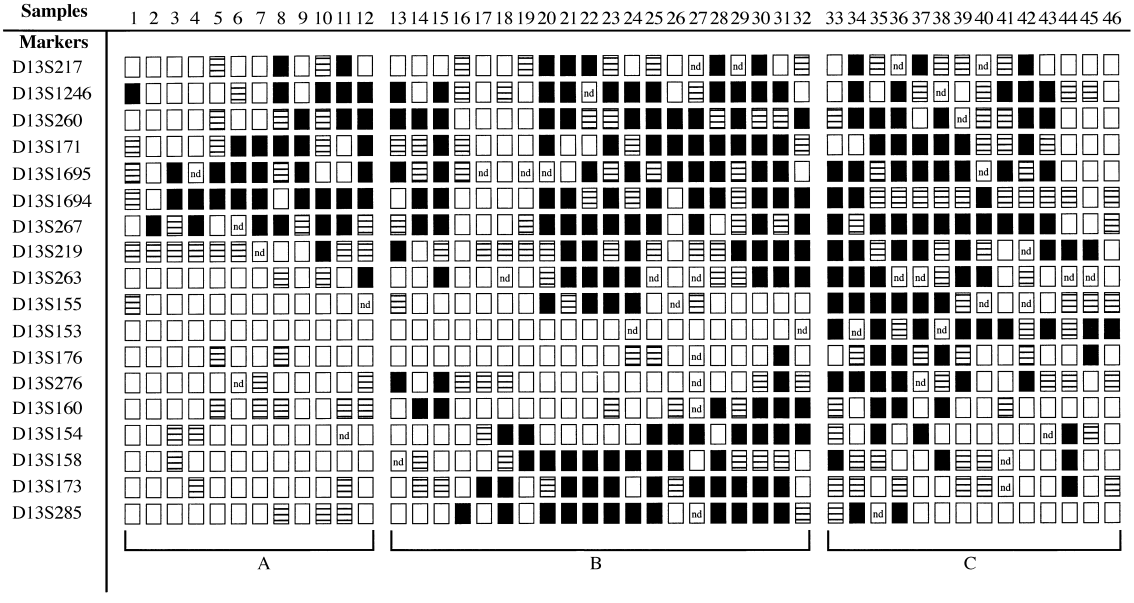

Figure 1. Deletion mapping of chromosome 13q in 46 breast tumours. Schematic representation of loss of heterozygosity (LOH): solid boxes show LOH, open boxes show retention of heterozygosity and cross-hatched boxes are homozygous for the given marker. nd = not determined. Case numbers are shown at the top. The data were obtained by polymerase chain reaction (PCR) with the markers listed on the left. Tumours in group A are indicative of LOH at 13q12-q13, in group B at 13q12-q13 as well as 13q31-q34, and in group C the retinoblastoma gene is involved in the tumours, as well as the two regions depicted in groups A and B.

the *RB1* region. LOH at the *RB1* region with retention of heterozygosity at 13q12-q13 was observed in two tumours (nos 45 and 46). Twelve tumours showed LOH at 13q12-q13 and no LOH with any other marker. Three tumours in group A (nos 2, 3, and 4) showed selective deletions restricted to markers distal to *D13S171* and proximal to *D13S263*, indicating the involvement of a gene in the 13q12-q13 region telomeric to *BRCA2*.

The results of the deletion mapping at 13q31-q34 are complex and suggest the possibility that more than one gene involved in the development of tumours in breast tissue could be located in this region. The majority of the tumours in group B indicate the location of one or more susceptibility genes, telomeric to marker *D13S154* (nos 16–24). The deletion mapping results of tumour nos 13, 14 and 15 suggest another target region proximal to marker *D13S154* and telomeric to *D13S176*.

Association with clinico-pathological variables

The results of the chi-square analyses of association of LOH at chromosome 13q with clinico-pathological variables are shown in Table 2. A significant association was found between LOH and high S phase fraction ($P < 0.0001$) and aneuploidy ($P = 0.016$). No association was found between LOH and axillary lymph node involvement, tumour size, ER content, PgR content, tumour type, or patient's age at diagnosis. Similar results were obtained when the three target regions depicted in Figure 1, i.e. 13q12-q13, the *RB1* region and 13q31-q34, were analysed separately, with two exceptions: a significant association was found between LOH at 13q12-q13 and low PgR content ($P = 0.031$) and no association was observed between LOH at *RB1* region and high S phase fraction. The same results were obtained when markers *D13S153*, *D13S155* and *D13S176* were grouped, or the intragenic marker for *RB1* (*D13S153*) was analysed separately.

Table 2. Chi-square analysis comparing loss of heterozygosity (LOH) at 13q *in sporadic breast tumour DNA with other categorised prognostic variables*

Variable	LOH	No LOH	*P*
All	71	68	
Node status			
Negative	34	40	
Positive	37	28	0.196
Tumour size (cm)			
≤ 2	27	35	
> 2	43	33	0.128
No information	1	0	
Histological type			
Ductal	63	60	
Lobular	8	8	0.927
ER (fmol/mg protein)			
≥ 10	46	48	
< 10	20	17	0.598
No information	5	3	
PgR (fmol/mg protein)			
≥ 25	33	42	
< 25	32	22	0.087
No information	6	4	
Ploidy			
Diploid	15	26	
Aneuploid	45	30	0.016*
No information	11	12	
S phase (%)			
< 7	20	39	
≥ 7	36	11	< 0.0001†
No information	15	18	
Age (years)			
< 50	21	18	
≥ 50	50	50	0.684

*95% confidence interval (CI), †99.9% CI. ER, oestrogen receptor; PgR, progesterone receptor.

Survival analysis

Early recurrence was found to be more common among patients with tumours exhibiting allele loss at one or more markers at chromosome 13q than among the patients with tumours without LOH ($P = 0.013$). This was followed by a separate analysis of the three target regions. The disease-free analysis revealed that patients with tumours with LOH at 13q12-q13 had a significantly earlier recurrence of the disease ($P < 0.001$, Figure 2). There was also a significant prognostic separation of overall survival curves for patients with tumours with and without LOH at 13q12-q13 ($P = 0.031$, data not shown). After 5 years of follow-up the overall survival rate was approximately 60% among patients with tumours showing LOH at chromosome 13q, and 78% among patients whose tumours retained heterozygosity. No significant difference was observed in survival or disease-free curves for patients with LOH at the *RB1* region or at 13q31-q34.

Multivariate analysis

The independent prognostic value of LOH at 13q12-q13 in breast cancer was evaluated using multivariate analysis (Table 3). In the series studied here, patients with tumours displaying LOH at this region have approximately a 3-fold increase in relative risk of mortality ($P = 0.045$). A similar risk was detected for lymph node involvement and high S phase fraction. The patients with LOH at 13q12-q13 in tumours

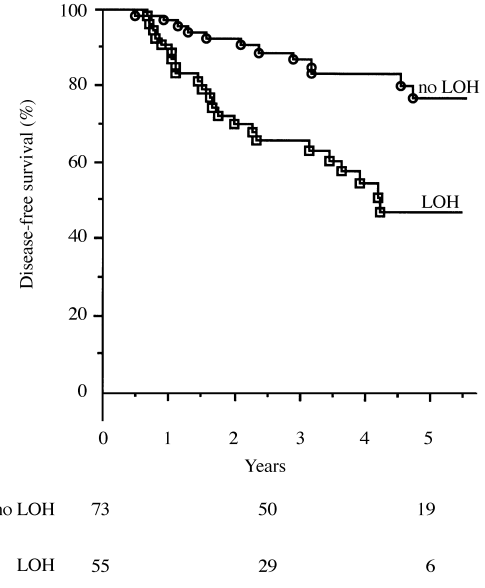

Figure 2. Disease-free survival in relation to loss of heterozygosity (LOH) at 13q12-q13 ($P<0.001$). *The number of patients at risk at time 0, at 2.5 years and 5 years is shown for both categories.

Table 3. Multivariate analysis (proportional-hazard (Cox) regression) of disease-free and overall survival in patients with breast cancer

	Disease-free survival ($n = 97$)			Overall survival ($n = 106$)		
Factor	Univariate *P* value	Multivariate *P* value	RR (with 95% CI)	Univariate *P* value	Multivariate *P* value	RR (with 95% CI)
13q12-q13 LOH	< 0.001	0.003	3.9 (1.6–9.4)	0.031	0.045	2.6 (1.0–6.7)
Node status	0.002	0.009	2.8 (1.3–5.9)	0.074	0.029	2.5 (1.1–5.6)
S phase fraction	< 0.001	0.033	2.3 (1.1–5.1)	0.002	0.052	2.5 (1.0–6.1)

RR, relative risk of death or recurrence; *n*, patients with a complete set of prognostic data; CI, confidence interval; LOH, loss of heterozygosity.

also had a 4-fold increase in relative risk of recurrence compared with other patients ($P = 0.003$). The addition of ER content or tumour size to the model did not affect the results of the multivariate analysis. The other regions on 13q, i.e. the *RB1* region and 13q31-q34 were also tested in the model, but did not show any prognostic value.

DISCUSSION

This study on sporadic breast tumours showed a high frequency of deletions at chromosome 13q. The deletion mapping suggests two separate targets of LOH in addition to the *RB1* gene. These are chromosome regions 13q12-q13 and 13q31-q34. The association of LOH with high S phase fraction and aneuploidy suggests that one or more genes located at 13q are involved in the control of cell proliferation and maintenance of genome stability. Survival analysis showed that LOH at 13q12-q13 has independent prognostic value and the patients with tumours where LOH was detected in this region are at a 3–4-fold increased relative risk of mortality and recurrence of cancer.

LOH at chromosome 13q12-q13, including the *BRCA2* gene, was observed in 43% of the breast tumours examined in this study. It should be emphasised that tumours from *BRCA2* mutation carriers were excluded from the study (see Material and Methods). The high frequency of LOH in sporadic tumours is in accordance with previously published results [15, 17, 21, 23] suggesting that loss of function of *BRCA2* or another gene located near to it might be important for breast tumour development. It would be of interest to analyse tumours showing LOH at 13q12-q13 with Brca2 specific antibodies to evaluate if expression of the *BRCA2* gene is reduced. Mutations in the *BRCA2* gene seem to be rare in sporadic tumours [24], making the possibility of another candidate gene in its vicinity more likely. *BRUSH*-1, that lies proximal to *BRCA2* at chromosome 13q12-q13, has been implicated in breast cancer [35]. However, additional means of inactivation of the *BRCA2* gene have not been excluded. The selective deletion observed in tumour nos 2, 3 and 4 indicates that a region telomeric to *BRCA2* but proximal to *RB1* harbours a gene that is a target for LOH.

LOH at 13q14, determined by one marker within the *RB1* gene, was 21%. This low frequency of deletion at the *RB1* region in sporadic tumours is in agreement with most other reports [22, 23, 36], although higher frequencies have also been observed [15, 17]. The *RB1* gene is a TSG shown to be deleted or mutated in many tumour types. However, *RB1* fails to reverse the malignant phenotype of breast cancer cell lines, suggesting that its loss does not account for breast tumorigenesis [37]. This is supported by observations of a lack of association between LOH at *RB1* and loss of pRb expression in breast tumours [22]. Loss at 13q12-q13 was observed in 88% of the tumours with LOH at the *RB1* region. The results support the idea that the *RB1* gene is deleted as a consequence of the loss of the 13q12-q13 region. However, the possibility that the *RB1* gene is inactivated in a subset of tumours cannot be excluded and is supported by the selective deletion determined in two tumours (nos 45 and 46) in group C, Figure 1.

LOH analysis telomeric to the *RB1* gene suggested an LOH target region at chromosome 13q31-q34, as 32% of the tumours showed LOH in this region. The distance between the five markers mapping to this region is 11 cM or more, so a large part of the chromosome is being considered. An aphidicoline sensitive fragile site is known at chromosome 13q32 [38]. LOH analysis at chromosome 13q31-qter has not been previously published for breast tumours, but the 13q32-qter region has been suggested as a preferential site of chromosome loss in head and neck squamous cell carcinomas [39]. Our analysis showed a significant association between LOH at 13q31-q34 and high S phase fraction and aneuploidy. No association was found with other clinical–pathological factors, with survival or disease-free survival. Further studies are needed to explore the possibility of this region harbouring a gene(s) of importance for breast cancer development or whether our results merely reflect the existence of an unstable chromosome region.

In an attempt to provide markers of early diagnosis of cancer and prognosis, the deletion results were compared with classical parameters of tumours. We found that LOH at 13q12-q13 was most frequent in tumours with low PgR content, high S phase fraction and aneuploidy. In a large LOH study conducted by Tsukamoto and colleagues [21] an association between low PgR content and LOH at 13q12-q13 was found, but this association was not observed in other studies [14–17]. Our results, showing an association with high S phase fraction and aneuploidy, are in line with other results [15, 17, 23] and suggest that loss of a gene(s) on 13q12-q13 can affect the control of cell proliferation and the maintenance of genome stability. Recently published studies have implicated *BRCA2* in the maintenance of genome stability [40]. In light of the fact that *BRCA2* mutations are rare in sporadic breast tumours [24], the question arises whether it can be inferred from the association of LOH at or near *BRCA2* with aneuploidy in this study that the remaining copy of *BRCA2* may not be sufficient for the maintenance of the integrity of the genome.

Survival analysis showed that patients with tumours where LOH at 13q12-q13 was detected have significantly worse prognosis than patients where LOH is not observed. Deletions at this region were of independent prognostic value. No differences in survival or recurrence-free survival were observed when patients were grouped according to LOH at

the *RB1* region or at 13q31-q34. Our results are in accordance with the results of van den Berg and associates [23] who also found a strongly significant correlation between LOH at the *BRCA2* region and a shortened recurrence-free survival and a less evident correlation for cases with LOH at *RB1*. An alteration of a target gene at 13q12-q13 seems a prognostic factor of clinical importance. Identification of the gene may be useful in the development of new biomarkers for evaluating breast cancer progression.

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