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Loss of Heterozygosity at Chromosome 7q in Human Breast Cancer: Association with Clinical Variables

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Abstract. *In this study 238 human primary breast cancers were analysed with 9 polymorphic microsatellite markers specific to region 7q21-q35 on chromosome 7. LOH was observed at one or more marker in 82 cases or (34%). The deletions were evenly distributed throughout the region. Patients were divided into two groups according to whether LOH was observed in their tumours or not, and tested for association with overall survival, the clinicopathological features: steroid receptor content, tumour size, node status, DNA ploidy and S-phase fraction, and LOH at other chromosomal regions. An association was found between 7q LOH and high S-phase fraction. An association was found between LOH at 7q and LOH at lp, 3p, 9p, 13q and 17q. These results suggest the location of a putative tumour suppressor gene at chromosome 7q21-q35 that, in combination with other deletions, might enhance tumour growth.*

Breast cancer is the most prevalent malignancy in women in the Western World (1). The incidence is rising and it is estimated that one of every eight women in the United States of America will get this disease in her lifetime (2). Prognostic variables have been increasingly used in breast cancer treatment to separate patients into two groups: a low-risk group, with low probability of recurrence where the side effects of any treatment would be worse than its benefits and a high-risk group with a substantial risk of recurrence and treatment would most likely be beneficial. The standard prognostic factors used today are tumour size, histopathological classification, histologic and nuclear grade, estrogen-receptor and progesterone-receptor status, and more recently also DNA ploidy and S-phase fraction. These conventional prognostic variables are considered as the phenotype which is the consequence of changes in the genome of the tumour cells (3). At present, there is an increased interest in looking for reliable prognostic factors in the genome of the tumour cells. Some studies have shown that mutations in the genome of tumour cells are of independent prognostic value.

Multiple somatic mutations can be detected in the genome of breast tumour cells. It is believed that these mutations have an impact on genes, that among other things, affect cell growth, proliferation, differentiation and programmed cell death. Deletions are the most commonly identified type of mutation in breast cancer. Deletions are observed in the tumour genetic material as loss of heterozygosity (LOH) and are thought to reveal the presence of a tumour suppressor gene within affected regions (4,5) .

Cytogenetic studies have shown deletions of chromosome 7q in secondary leukaemia and myeloproliferative disorders (6,7), squamous cell carcinomas of the head and neck (8), prostate carcinomas, colorectal carcinomas and testicular germ cell tumours (9). Both a Southern blot study and polymerase chain reaction (PCR) of restriction fragment length polymorphism (RFLP) studies have shown LOH on 7q in myeloid disorders (10,11) and in gastric carcinomas (12). Mapping studies with microsatellite markers have shown frequent LOH at 7q31.1-q31.2 in prostate carcinomas (13), squamous cell and colon carcinomas (14), ovarian carcinomas (15) and breast cancer (16).

The results of LOH studies on 7q in breast cancer have been inconsistent, the deletion frequency varying from 0-84% (16,17,18,19,20). One of these studies reports a strong association with overall survival and metastatic free survival,

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suggesting LOH at the MET locus on 7q as a significant independent prognostic factor (20). A Southern blot study on both primary breast tumours and relapses showed that there was no significant difference in the deletion frequency between these two groups of samples so the conclusion was drawn that LOH on 7q was an early event in breast cancer (21).

Ogata *et al* (22) studied non-tumorigenic immortalised human fibroblast cell lines and found that these cells had chromosomal alterations on chomosome 7q in common. When chromosome 7 was introduced into these cells they reduced the proliferation rate and resembled cells in senescence. Introduction of other chromosomes did not have this effect. A similar experiment demonstrated that insertion of an intact chromosome 7 into a mouse-derived squamous cell carcinoma cell line delayed the onset of tumours two-three fold in nude mice and could occasionally repress it totally, compared with cells without chromosome 7 insertion (23).

The aim of our study was to map LOH on 7q21-q35 in sporadic primary breast cancer using microsatellite markers and thus localize a putative tumour suppressor gene. Furthermore, we compared tumours with and without LOH at 7q with respect to conventional clinicopathological features (steroid receptor content, tumour size, node status, DNA ploidy and S-phase fraction) and LOH at other chromosomal regions.

Materials and Methods

Patients and tumour material. Fresh biopsies from primary breast tumours are routinely sent to our laboratory for estrogen and progesterone receptor (ER and PgR) analysis. Blood samples from the patients are collected in EDTA and if not processed immediately, tumours and blood were quick frozen at -70°C. All information about the tumours, *e.g.*, size, type, and node status, was recorded by the Department of Pathology, National Hospital of Iceland. 238 primary breast cancer samples were analyzed. Each of these tumour samples was matched with blood from the same individual for reference. 128 of these patients were lymph node negative and 110 were positive for one or more lymph nodes. The average age of the patients at diagnosis was 62 years.

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 pulverized primary tumour tissue (24). DNA was extracted from blood lymphocytes and matched with tumour DNA as reference (25). 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 40ng of genomic DNA, 5 pmol of the forward and reverse primers, 2.5 nmol of each dNTP, 0.5 units DynaZyme polymerase. A hot start was used by adding the enzyme during the first cycle at about 72°C. The samples were amplified in 35 cycles composed of 30 seconds of denaturation at 94°C, 30 seconds annealing at 55°C and finally 60 seconds of extension at 72°C. The microsatellite markers used (D7S492, D7S518, D7S515, D7S471, D7S501, D7S523, D7S522, D7S480, D7S500) were obtained from the Nordic primer resource in Uppsala (C. Wadelius, Department of Clinical Genetics, University Hospital, Uppsala, Sweden) and Research Genetics (Huntsville, AL, USA).

Table I. *Information about the markers used in this study, number of samples tested with each marker and frequency of LOH at each marker.*

Marker	distance cM	Cytological location	number of samples	het (%)	LOH(%)
D7S492		7p15-q22	216	152 (70)	18 (12)
	14				
D7S518		7q21-q31	226	198 (88)	21 (11)
	1				
D7S515		7q21-q31	222	186 (84)	26 (14)
	7				
D7S501		7q31	218	157 (72)	21 (13)
	5				
D7S523		7q31	216	173 (80)	19 (11)
	2				
D7S471*		7q31	204	174 (85)	20 (11)
D7S522		7q31	221	172 (78)	17 (10)
	1				
D7S480		7q31-q35	217	141 (65)	15 (11)
	15				
D7S500		7q31-q35	218	184 (84)	26 (14)

Information of cytological locations and distances between markers was obtained from the Genome Data Base, Baltimore.
* According to GDB Linkage-average map the D7S471 marker is located approx. 6 cM telomeric to marker D7S501.

The PCR products were subjected to denaturing gel electrophoresis in 6.5% polyacrylamide, 8M urea denaturating gel, transferred to a positively charged nylon membrane, Hybond-N+ (Amersham, Aylesbury, UK) and baked for at least 2 hours at 80°C. The non-radioactive detection method to visualize the PCR products has been described previously (26). Autoradiograms were inspected visually by at least two reviewers, comparing the intensity of alleles from normal and tumour DNA. Absence or significant decrease of one allele in the tumour compared to the normal reference sample was considered as LOH (Figure 1).

Flow cytometry. A representative paraffin block containing adequate amounts of tumour cells was selected from each tumour. The DNA content of tumour cells was analyzed using a single cell suspension prepared according to a modified method described by Jonasson and Hrafnkelsson (27). Nuclear DNA was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). A total of 20.000 cells was analyzed for each tumour. The Cell Fit system (Becton Dickinson) was used for S-phase analyses and calculated with a planimetric method. The tumour ploidy was defined as diploid if only one stem cell population was detected on the histogram and as aneuploid if two or more stem cell populations were detected.

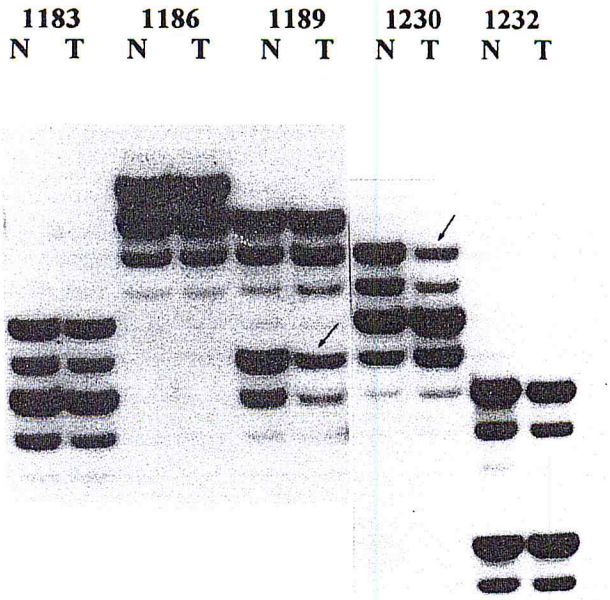

Figure 1. *Autoradiogram of a dinucleotide repeat polymorphism in five matched normal (N) and tumour (T) tissues from breast cancer patients. The case numbers are indicated above each autoradiogram. The microsatellite marker D7S500 was used in PCR amplification and the amplified products separated in 6.5% polyacrylamide, 8M urea denaturing gel. Case 1186 is homozygous. LOH is seen in cases 1189 and 1230. Retension of heterozygosity is seen in cases 1183 and 1232.*

Statistical analysis. A χ^2 test was used to assess the relationship between LOH at 7q and the clinico-pathological variables. Prognostic factors were categorised using commonly applied cut-off points (Table II). Survival curves were calculated according to the method of Kaplan and Meier. Tests of difference between curves were made with the log-rank test for censored survival data. The Statistica/Mac package (Statsoft, Tulsa, OK) was used for the statistical analyses.

Association with LOH at other chromosomal regions. Some of the tumour/normal pairs used in this study have been screened for LOH at other chromosomal regions in our laboratory. We used χ^2 analysis to assess if there was a relationship between LOH at 7q and genetic changes at 1p, 3p, 6q, 9p, 11q, 13q, 16q, 17p and 17q. Results from the studies on chromosomes 3p, 6q, 9p, 11q and 16q have been published (28, 29, 30, 31, 32). Manuscripts describing the results of the studies on chromosomes 1p, 13q, 17p and 17q are in preparation.

Results

238 primary breast tumours were analysed for LOH using 9 microsatellite markers mapping to 7q21-q35. LOH at this region was observed in 82 of the 238 cases analysed (34%). Seven tumours with deletions showed LOH at all informative markers, indicating a total loss of the 7q21-q35 region. Forty tumours showed LOH at two or more markers. No one marker showed a distinctively high frequency of LOH. The

Table II. *Chi-square analysis comparing loss of heterozygosity (LOH) at 7q in tumour DNA to categorized prognostic factors.*

Variable	LOH/TOTAL	%	p
All	82/238	34	
Node status			
negative	47/128	37	
positive	35/110	32	0.43
Tumour size			
< 2 cm	29/98	30	
≥ 2 cm	53/139	38	0.17
no information	0/1		
Histological type			
ductal	78/214	36	
lobular	4/23	17	0.068
no information	0/1		
Estrogen receptors			
fmol/mg protein			
≥ 10	56/161	35	
< 10	22/66	33	0.83
no information	4/11		
Progesterone receptors			
fmol/mg protein			
≥ 25	38/119	32	
< 25	39/105	37	0.41
no information	5/14		
Ploidy			
diploid	23/78	29	
aneuploid	50/128	39	0.16
no information	9/32		
S-phase			
< 7%	31/110	28	
≥ 7%	38/80	48	0.0063**
no information	13/48		
Age			
< 50	21/82	26	
≥ 50	61/156	39	0.037*

* = 95% CI. ** = 99% CI. (CI= Confidence interval).

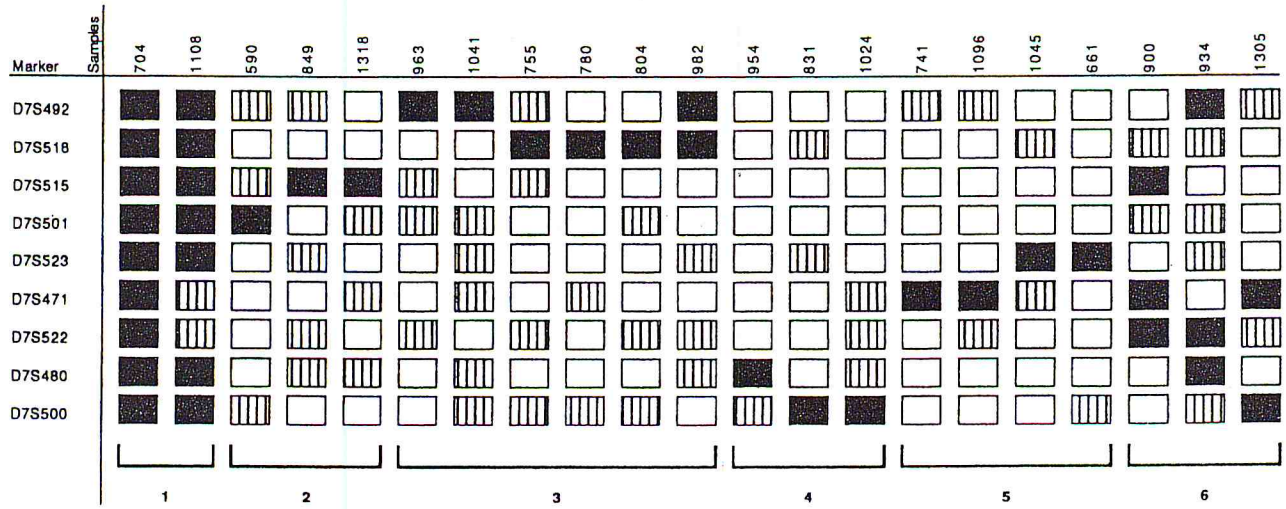

Figure 2. *Pattern of LOH on chromosome 7q for 21 representative breast cancer samples. Group 1 shows complete loss of 7q. Group 2 is consistent with smallest common deletion region (SCDR) between marker D7S518 and D7S501. Group 3 is consistent with SCDR between markers D7S492 and D7S518. Group 4 is consistent with SCDR distal to marker D7S480. Group 5 is consistent with SCDR between markers D7S523 and D7S471. Group 6 shows complex LOH.* ■ *LOH,* □ *Heterozygosity retained,* ▥ *Homozygous.*

frequency of LOH varied from 10-14% for each individual marker (Table I). Figure 2 shows the deletion pattern of 21 representative tumours, 19 show partial or interstitial deletions which an attempt has been made to categorise. No smallest common deletion region (SCDR) could be defined, but the tumours were categorized into four groups according to the interstital deletions observed (Figure 2).

Results from the χ^2 analysis comparing LOH on 7q with clinico-pathological factors are shown in Table II. A significant association was found between LOH at 7q and high S-phase fraction and age, deletions at 7q being more common in older women.

Survival analyses with a median of 3.4 years follow up showed no significant difference in survival between breast cancer patients with tumours with deletions at 7q and other patients.

The results from the χ^2 analysis comparing LOH at 7q with deletions at other chromosomes are shown in Table III. A significant association was found between LOH at 7q and deletions at 1p, 3p, 9p, 13q and 17q but not at 6q, 11q, 16q, and 17p.

An interesting observation was made in one case where we were able to examine the primary breast tumour and also an axillary relapse, excised four years after the diagnosis of the primary tumour. No LOH at 7q was observed in the primary tumour although deletions were observed at other chromosome regions. However, deletion at 4 markers at 7q was observed in the relapse.

Discussion

In this study 34% of 238 primary breast cancer tumours were

Table III. *Chi-square analysis was used to test for significance between LOH at 7q and LOH at nine other chromosomal regions.*

Chromosome region	Sample size	Number of patients: LOH	7qLOH	7qLOH and region	p
1p	225	97	78	44	0.0034**
3p	134	36	50	20	0.0081**
6q	154	62	61	29	0.14
9p	151	52	53	25	0.015*
11q	62	22	22	8	0.91
13q	135	54	48	26	0.013*
16q	162	108	58	42	0.25
17p	84	37	29	13	0.92
17q	75	21	26	11	0.044*

The number of cases tested at 7q that were also analyzed at other regions is shown in the table as sample size for each region. The number of cases with LOH at each specific region is shown. The number of samples with LOH detected both at 7q and at each other chromosomal region is also shown.
* = 95% CI. ** = 99% CI

found to have LOH at 7q21-q35. Our results are similar to studies where Southern blots were used to determine deletion frequencies *i.e.* 41% (20), 36% (21) and 27% (19). However in our study, the frequency of LOH at 7q is low for each individual marker tested, or 10-14%. A much higher frequency, 84%, was observed in a study using microsatellite markers involving 31 primary breast cancer tumours where 90% of the tumours were stage II or more advanced (16). The tumours in our study were less advanced than the tumours in the other studies and could account for the discrepancy in deletion frequency. Different methodology and tumour heterogeneity could also explain some of the differences between these studies.

The deletions were evenly distributed throughout the region tested. No single marker within the region showed an especially high deletion frequency compared with the others and these results cannot exclude the presence of more than one putative tumour suppressor gene within the region. The microdeletions were catagorised into four main regions, shown in Figure 2. Zenklusen *et al* (16) reported that the region around D7S522 is most commonly deleted in primary breast cancer, with 9 of 11 informative samples showing LOH and the smallest common deleted region of lcM. A similar trend cannot be seen in our results. The partial deletions detected (Figure 2) do not suggest SCDR close to this marker and the zebra pattern seen in some samples is hard to explain.

LOH at 7q was compared with various prognostic variables. No association was found with the conventional prognostic variables, such as positive node status, tumour size or low estrogen or progesterone receptor content (Table II). A statistically significant association was found between 7q LOH and high S-phase fraction. S-phase fraction is an important prognostic factor in primary breast cancer, high S-phase fraction being associated with poor prognosis (3). Sphase has been shown to be the most important prognostic factor in node negative breast cancer (33). A gene at 7q could possibly have a restraining effect on the rate of cell proliferation, and the loss of it would lead to a higher S-phase fraction. Another possibility is that a high S-phase fraction leads to an unstable genome, leading to LOH at many chromosomal regions and the cells with 7q LOH would be selected due to growth advantage. This putative growth advantage cannot be strong since only a low proportion of tumours exhibit LOH at 7q.

No difference was found in overall survival between patients with tumours where 7q LOH was observed and the other patients. The median follow up time is similar to the study that reports a poorer overall survival in patients with LOH at 7q (20). That same study also reports a significant association between LOH at 7q and metastasis free survival and LOH at 7q was determined to be an independent prognostic factor. We do not have the necessary data regarding metastasis free survival to confirm this finding but we did not find LOH at 7q to be an independent prognostic factor. However, in studies involving the same set of samples, LOH at 3p, 6q, 13q, 17q (28, 29, unpublished data) has been shown to be an independent prognostic factor of poor overall survival. The results indicate that the combination of LOH at 7q with other tumour suppressor gene deletions might enhance tumour growth.

LOH on 7q21-q35 was found to be cumulatively high in primary breast cancer and the deletions were evenly distributed throughout the region. An association was found between 7q LOH and high S-phase fraction and older age of diagnosis. No difference was observed in overall survival between patients with tumours with and without LOH at 7q. LOH at 7q was not found to be an independent prognostic factor. An association was found between LOH on 7q and LOH at lp, 3p, 9p, 13q, and 17q. The results suggest a putative tumour suppressor gene in the region tested, that in combination with other deletions, might enhance tumour growth.

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