High Frequency of LOH at Chromosome 18q in Human Breast Cancer: Association with High S-Phase Fraction and Low Progesterone Receptor Content

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Abstract. Human primary breast cancers were analysed for somatic loss of heterozygosity (LOH) at chromosome 18 with 15 polymorphic microsatellite markers. LOH was observed in 148 of the 228 cases analyzed, (65%). Three smallest common deletion regions (SCDR) were detected on the long arm of chromosome 18. The marker D18S51 at the region 18q22 showed the highest LOH (42%). Tumors with and without LOH at 18q were tested for association with clinico-pathological features of the tumors, such as estrogen and progesterone receptor content, age at diagnosis, tumor size, node status, histological type, S-phase fraction, DNA ploidy and LOH at other chromosomal regions. A significant association was found between LOH at 18q and high S-phase fraction (99.9% confidence interval) and low progesterone receptor content (99% confidence interval). Furthermore, an association was found between LOH at 18q and LOH at 1p, 7q, 9p, 13q and 17q. We conclude that there are three separate LOH target regions at chromosome 18q, and that inactivation of one or more genes at these regions might be important for human breast carcinogenesis.

Breast cancer incidence has been increasing worldwide in recent decades. Nearly one in every eight women in the USA will get this disease in her lifetime (1). It is the most prevalent malignancy in the Western world and has the highest death rate of any malignancy (2). Chromosomal alterations, usually deletions, could imply that inactivation of tumor suppressor genes plays an important role in tumorigenesis. LOH at 18q has been reported in various cancer types, such as breast, colorectal, prostatic, ovarian, lung, renal cell, gastric and pancreatic carcinomas, and osteosarcoma, suggesting a tumor suppressor gene or group of genes at this chromosomal arm (3,4,5,6,7,8). Frequent LOH in breast cancer has been detected on chromosome arms 1p, 1q, 3p, 6q, 7q, 9p, 11p, 11q, 13q, 16q, 17p, 17q and 18q (9-19).

The results of LOH studies at 18q in breast cancer have been inconsistent, the deletion frequency varying from 15% to 62% (3,19,20,21,22). Three putative tumor suppressor genes have been cloned and identified from chromosome 18q21.1. These genes are the DCC (deleted in colorectal cancer) (4,23) and two Mad (Mothers against dpp)-related genes, DPC4 (deleted in pancreatic carcinomas, locus 4) and MAD2R (MAD-related gene 2) (8,23,24). The detected loss of heterozygosity in breast tumors indicates that the inactivation of a tumor suppressor gene or genes located at chromosome 18q21.1 may play a role in human breast carcinogenesis (3,19,20,21,22). Loss of the DPC4 gene is suspected as a major cause of pancreatic carcinoma and DCC is an additional candidate in colorectal cancer (4,8,23,24).

The aim of our study was to map LOH at 18q in sporadic primary breast cancer and thus localize a putative tumor suppressor gene. We compared tumors with and without LOH at 18q with respect to clinico-pathological features to determine whether LOH at 18q is of prognostic value for breast cancer patients. We compared LOH at 18q with deletions at other chromosomal arms in an attempt to determine a common pathway for genetic events in breast tumors.

Materials and Methods

Patients and tumor material. In all, 228 fresh biopsies from primary breast tumors were analyzed. Blood samples from the patients were collected in EDTA and if not processed immediately, tumors and blood were quickly frozen at -70°C. The blood samples were screened for the 999del5 BRCA2 mutation that has been detected in 8.5% of Icelandic breast cancer patients (25). Samples from patients carrying the 999del5 mutation were excluded from the study.

DNA extraction and analysis. Standard procedures were used for extracting tumor DNA, either from the nuclear pellet remaining after cytosol removal for estrogen and progesterone receptor (ER and PgR) analysis or pulverized primary tumor tissue (26). DNA was extracted from blood lymphocytes using the salting out method (27). The DNA was subjected to PCR amplification using DynaZyme polymerase

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(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 mM of each dNTP, 0.5 units of Dynazyme polymerase. 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. A hot start was used by adding the enzyme during the first cycle at about 72°C after a preincubation time of 5 minutes at 94°C. The microsatellite markers used (D18S54, D18S66, D18S67, D18S46, D18S450, D18S470, D18S474, DCC, D18S69, D18S68, D18S51, D18S61, D18S488, D18S58, D18S70) were obtained from Research Genetics (Huntsville, AL, USA). Marker DCC is located within the DCC gene (28). Distances between the markers were obtained from Gyapay, et al (29).

The PCR products were subjected to electrophoresis in 6.5% polyacrylamide, 8M urea denaturing gels, transferred to a positively charged nylon membrane, Hybond-N+ (Amersham, Aylesbury, UK) and baked for at least 2 hours at 80°C. The nonradioactive detection method used to visualize the PCR products has been described previously (30). Autoradiograms were inspected visually by at least two reviewers, comparing the intensity of alleles from normal and tumor DNA. The absence of a significant decrease of one allele in the tumor compared to the normal reference sample was considered as LOH (Figure 1). Due to normal cell contamination in the tumor samples, there is a risk of a conservative estimate of LOH. S-phase fraction and DNA ploidy were measured using a FACScan flow cytometer (Beckton-Dickinson).

Statistical analysis. A Chi-square test was used to assess the relationship between LOH at 18q and the clinicopathological variables. The cut-off points used to categorise the prognostic value are shown in Table II. Survival curves were calculated according to the Kaplan-Meier method. Tests of difference between curves were made with the log-rank test for censored survival data. Survival tools for StatView (Abacus Concepts, Inc., Berkeley, CA) was used for the statistical analyses.

Association with LOH at other chromosomal regions. Many of the tumor/normal pairs used in this study have been screened for LOH at other chromosomal regions. We used Chi-square analysis to assess if there was a relationship between LOH at 18q and LOH at 1p, 3p, 6q, 7q, 9p, 11p, 11q, 13q, 16q, 17p, and 17q. Results from the studies on chromosome 1p, 3p, 6q, 7q, 9p, 11p, 11q and 16q have been published (12,13,31-35). Manuscripts describing the results of the studies on chromosome 13q, 17p and 17q are in preparation.

Results

In all, 228 primary breast tumors were analysed for LOH using 15 microsatellite markers mapping to chromosome 18. LOH with at least one marker was observed in 148 of the 228 cases analysed (65%). There were 15 tumors with LOH at all markers tested, indicating loss of the total chromosomal arm, 113 tumors with complex LOH and 20 tumors with microdeletions. LOH for individual markers ranged from 26% to 42% (Table I). The highest percentage of LOH (42%) was detected by the marker D18S51 and a high frequency was also detected by the markers D18S70 (37%), D18S67 (35%), D18S54 (34%) and D18S58 (34%) (Table I).

The pattern of LOH is consistent with the smallest common deletion region (SCDR) at 18q21, which is between markers D18S470 and D18S68 (Figure 2, group 1). This region is less than 5 cM and the DCC gene is within this region (Table I).

Figure 1. Autoradiograms of PCR products with 4 microsatellite markers in matched normal (N) and tumor (T) tissues from 2 breast cancer patients. The amplified products were separated in 6.5% polyacrylamide, 8M urea denaturing gels. Case numbers are shown at the top. Symbols to the left indicate the markers. Numbers to the right indicate the size (in nucleotides) of the DNA product. LOH indicated by arrows can be seen in both cases with D18S69. Retained heterozygosity is observed with markers D18S68, DCC and D18S474. Both cases are in group 1. Figure 2.

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Table I. Markers used and frequency of LOH.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Distance from centromere (cM)</th>
<th>Location</th>
<th>Number of samples tested</th>
<th>Informative samples (%)</th>
<th>Tumors with LOH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D18S54</td>
<td>45</td>
<td>18pter-p11</td>
<td>182</td>
<td>114 (63)</td>
<td>39 (34)</td>
</tr>
<tr>
<td>D18S66</td>
<td>11</td>
<td>18q12.1</td>
<td>193</td>
<td>155 (80)</td>
<td>48 (31)</td>
</tr>
<tr>
<td>D18S67</td>
<td>18q12.2-q12.3</td>
<td>195</td>
<td>135 (69)</td>
<td>47 (35)</td>
<td></td>
</tr>
<tr>
<td>D18S68</td>
<td>18q12.3</td>
<td>203</td>
<td>140 (69)</td>
<td>36 (26)</td>
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</tr>
<tr>
<td>D18S70</td>
<td>18q21.1</td>
<td>213</td>
<td>146 (69)</td>
<td>44 (30)</td>
<td></td>
</tr>
<tr>
<td>D18S74</td>
<td>18q21.1</td>
<td>189</td>
<td>161 (85)</td>
<td>44 (27)</td>
<td></td>
</tr>
<tr>
<td>DCC</td>
<td>5</td>
<td>18q21.1</td>
<td>223</td>
<td>192 (86)</td>
<td>63 (33)</td>
</tr>
<tr>
<td>D18S69</td>
<td>18q21.3-q21.3</td>
<td>202</td>
<td>138 (68)</td>
<td>43 (31)</td>
<td></td>
</tr>
<tr>
<td>D18S68</td>
<td>18q22.1</td>
<td>202</td>
<td>169 (84)</td>
<td>49 (29)</td>
<td></td>
</tr>
<tr>
<td>D18S51</td>
<td>18q22</td>
<td>198</td>
<td>179 (90)</td>
<td>76 (42)</td>
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<tr>
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<td>18q22.3</td>
<td>213</td>
<td>183 (86)</td>
<td>48 (26)</td>
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<tr>
<td>D18S48</td>
<td>18q22.3</td>
<td>191</td>
<td>161 (84)</td>
<td>48 (30)</td>
<td></td>
</tr>
<tr>
<td>D18S58</td>
<td>18q22.3-q23</td>
<td>205</td>
<td>154 (75)</td>
<td>53 (34)</td>
<td></td>
</tr>
<tr>
<td>D18S70</td>
<td>18q23</td>
<td>192</td>
<td>168 (88)</td>
<td>62 (37)</td>
<td></td>
</tr>
</tbody>
</table>

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

Figure 1 shows selected deletions in two breast tumors. The LOH is restricted to marker D18S69, while markers in the vicinity show retention of heterozygosity. Group 2 in Figure 2 suggests SCDR between markers D18S68 and D18S61. Group 3 in Figure 2 suggests SCDR telomeric to D18S58, a region of 15 cM.

Results from the Chi-square analysis comparing LOH at 18q with clinicopathological variables are shown in Table II. There was a significant association between LOH at 18q and high S-phase fraction (99.9% confidence interval) and between LOH at 18q and low PgR content (99% confidence interval). There was no significant association between LOH at 18q and the other parameters tested, i.e., ploidy, node status, histological type, ER content, tumor size and age at diagnosis.

Table III shows the Chi-square analysis comparing LOH at 18q with LOH at other chromosomal regions investigated in our laboratory. There was a significant association between LOH at 18q and LOH at the following chromosomal regions: 1p, 7q, 9p, 13q and 17q. There was no association between...
LOH at 18q and LOH at 3p, 6q, 11p, 11q, 16q and 17p.

Survival analyses with a median follow-up time of 4.2 years showed no significant difference in survival between breast cancer patients with tumors with deletions at 18q and the other patients tested in this study.

Discussion

In this study LOH at chromosome 18q was detected with at least one marker in 65% of the tumors examined. The elevated frequency of LOH with certain markers and the mapping of the smallest common deletion region (SCDR) suggest three regions of deletions at 18q21.1-q21.31, 18q22.1-q22.3 and 18q22.3-q23 indicating locations for candidate tumor suppressor genes. The chromosomal band 18q21.1, known to carry the putative tumor suppressor genes DCC and DPC4, is frequently deleted in breast cancer (3,21,22). Our results are in accordance with this finding. The 18q21.1 region is also deleted in other solid tumors such as osteosarcoma (36), colorectal (23), lung (37), gastric (38), prostatic (39) and renal cell carcinoma (40). The DCC gene encodes for a transmembrane protein with strong structural similarity to members of the superfamily of neural cell adhesion molecules.

Table III. Association of LOH at 18q and LOH at other chromosome regions.

<table>
<thead>
<tr>
<th>Region</th>
<th>p value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p</td>
<td>0.002**</td>
<td>218</td>
</tr>
<tr>
<td>3p</td>
<td>0.082</td>
<td>122</td>
</tr>
<tr>
<td>6q</td>
<td>0.13</td>
<td>144</td>
</tr>
<tr>
<td>7q</td>
<td>0.011*</td>
<td>218</td>
</tr>
<tr>
<td>9p</td>
<td>0.037*</td>
<td>149</td>
</tr>
<tr>
<td>11p</td>
<td>0.41</td>
<td>52</td>
</tr>
<tr>
<td>11q</td>
<td>0.77</td>
<td>61</td>
</tr>
<tr>
<td>13q</td>
<td>0.001**</td>
<td>131</td>
</tr>
<tr>
<td>16q</td>
<td>0.93</td>
<td>152</td>
</tr>
<tr>
<td>17p</td>
<td>0.069</td>
<td>80</td>
</tr>
<tr>
<td>17q</td>
<td>0.025*</td>
<td>87</td>
</tr>
</tbody>
</table>

n=number of samples tested. **=99% confidence interval. *=95% confidence interval.
and it is implicated in cellular differentiation and developmental processes (36). Its loss or reduction of expression could be a specific event in the development or progression of cancer (36). The putative tumor suppressor genes DPC4 and MADR2 are also located at 18q21.1 (8,23,24). They are both components in a transforming growth factor beta (TGF-β)-like signaling pathway (41). TGF-β is a potent inhibitor of cellular proliferation (42). Transfer of DPC4 into breast cancer cells has been shown to partially restore TGF-β-induced growth inhibition of tumor cells (43). Studies where loss was observed at DPC4 and MADR2 showed that inactivation of these genes may play a role in pancreatic, colorectal, breast and possibly other human cancers (8,23,24).

In addition to chromosome 18q21.1, the 18q23 region has been suggested to contain a suppressor gene relevant to breast carcinogenesis (22). Our mapping results support these observations, since we have located an SCDR at the 18q23 region. We found the highest frequency of LOH (42%) in the 18q22.1-q22.3 region, suggesting that a gene important in suppressing breast cancer could possibly be located in this region.

LOH at 18q was compared with various prognostic variables. A strong association was found between 18q LOH and high S-phase fraction. S-phase fraction is an important prognostic factor in primary breast cancer, a high S-phase fraction being associated with poor prognosis (44). Our results suggest that a gene at 18q could possibly have a restraining effect on the rate of cell proliferation, and the loss of it would lead to a high S-phase fraction. We found a significant association between LOH at 18q and low PgR content. However, no conclusion can be drawn from our studies regarding the functional relationship of 18q LOH and reduced expression of PgR. Survival analysis showed no difference in overall survival between patients with tumors with and without LOH at 18q. LOH at 18q was not found to be of prognostic value.

A number of studies have revealed that chromosomal deletions in human breast tumors may occur in preferred combinations, e.g., 1p and 3p (31), 7q and 1p (12), 3p and 6q (33), 9p and 6q (13), 13q and 17p (45,46), 11p and 17p (47). In order to determine whether LOH at 18q is involved in specific combinations of chromosomal deletions, we compared the results in this study with previous deletion studies done in our laboratory on the same tumor material. We found a significant association between LOH at 18q and LOH at 13q and 1p. There was a weak association between LOH at 18q and LOH at 7q, LOH at 9p and LOH at 17q. The association between LOH at more than one locus fits well with the clonal theory of cancer growth which proposes that an accumulation of multiple genetic alterations play an important role in tumorigenesis. Preferred combinations of tumor suppressor gene deletions might result in enhanced tumor growth.

No significant association was detected between 18q LOH and LOH at 3p, 6q, 11p, 11q, 16q and 17p, suggesting that deletions in some regions of the genome are independent events and reflect the multifactorial tumor progression of breast cancer.

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