High Incidence of Loss of Heterozygosity in Breast Tumors from Carriers of the BRCA2 999del5 Mutation

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ABSTRACT

Germ-line mutation in the BRCA2 gene confers an increased risk of breast cancer. An elevation of additional genetic defects in tumors of patients with germ-line mutation in the BRCA2 gene compared with sporadic breast tumors has been reported. To evaluate the nature of the difference, we did detailed mapping of chromosomes 1p, 3p, 6q, 11, 13q, 16q, 17, and 20q, using microsatellite markers. We found that the frequency of loss of heterozygosity was similar at some chromosomal regions in the BRCA2 999del5 and sporadic tumors but significantly different at others. These others include chromosomal arms 3p, 6q, 11p, 11q, 13q, and 17p. Loss of heterozygosity mapping suggests that the same chromosome regions are involved in both tumor groups but at elevated frequencies in BRCA2 999del5 tumors. This higher frequency of genetic aberrations could pinpoint genes that selectively promote tumor progression in individuals predisposed to breast cancer due to the BRCA2 999del5 germ-line mutation. Accumulation of somatic genetic changes during tumor progression may follow a specific and more aggressive pathway of chromosome damage in these individuals.

INTRODUCTION

A complex set of genetic alterations is involved in the etiology of breast cancer. The detected genetic abnormalities in breast tumors are amplification of oncogenes (MYC, ERBB2, and CCND1), mutation of the tumor suppressor gene TP53, and LOH from chromosomes 1, 3p, 6q, 7q, 8p, 11, 13q, 16q, 17, 18q, and 22q (reviewed in Refs. 1 and 2). The LOH may correspond to losses or inactivation of tumor suppressor genes. Furthermore, mutations of BRCA1 and BRCA2 genes have been implicated in inherited breast cancer (3–5). Somatic loss of the wild-type chromosome in tumors of BRCA1 and BRCA2 mutation carriers suggests that both alleles of the corresponding gene are inactivated in cancer, a pattern expected of a tumor suppressor gene (6–8). A germ-line mutation in the BRCA2 gene, termed 999del5, has been detected in Icelandic breast cancer families, and a common haplotype in the vicinity of the gene in these families suggests a founder effect (9–11). The BRCA2 999del5 germ-line mutation has been found in 8% of patients diagnosed with breast cancer in Iceland (11, 12). A genome-wide search for the chromosome changes in tumors of BRCA2 carriers has been done by CGH and demonstrated a higher frequency of aberrations at several chromosome arms compared with sporadic tumors (13). These results suggested a specific tumor progression pathway in patients predisposed to breast cancer due to a BRCA2 mutation. In this study, we have used microsatellite markers for more detailed mapping of somatic aberrations in breast tumors of BRCA2 999del5 carriers and compared changes in sporadic breast tumors at chromosome regions already shown to differ in the two groups by CGH (13). A profile of LOH has not been reported earlier on a set of tumors carrying a single specific mutation in BRCA1 or BRCA2 genes.

MATERIALS AND METHODS

Subjects. Breast cancer patients diagnosed in the years 1989–1997 were screened for the BRCA2 999del5 mutation. Male breast cancer was excluded from the study. Forty-six patients were positive for the BRCA2 999del5 mutation, of 541 unselected female patients analyzed. All tumors were primary invasive breast tumors, 487 of ductal and 54 of lobular histological type. A set of tumors negative for the BRCA2 999del5 mutation was used as the control group (number of tumors are given in Table 1). Part of this patient material has been used in our previous studies on LOH in human breast tumors at chromosome regions: 1p, 232 patients (14); 3p, 140 patients (15); 6q, 204 patients (16); 11, 116 patients (17); 13q, 139 patients (18); and 16q, 150 patients (19). These studies were done prior to the knowledge of the BRCA2 999del5 mutation, except the study on chromosome 13, where BRCA2 999del5 carriers were excluded.

Mutation and Microsatellite Marker Analysis. The BRCA2 999del5 mutation analysis is based on allele size difference (186-bp normal and 181-bp mutant allele) using forward (5′-ATGGTAAAGGGGGGACTA-3′) and reverse (5′-AACTGAGATCCAGGGT-3′) primers in a PCR analysis (11). Ten chromosome arms were analyzed with microsatellite markers to score for LOH: 1p, 3p, 6q, 11q, 13q, 16q, 17p, and 20q. The rationale for the inclusion of these chromosome regions was based on previous results by using CGH, showing significant difference in the frequency of aberration in BRCA2 carriers compared with controls (13). PCR primers used for the microsatellite marker analysis were (cytogenetic chromosome localization according to the Genome Data Base are given in parentheses): D1S243 (Ip36.3); D1S468 (Ip36.3); D1S214 (Ip36.3); D1S228 (Ip36.1); D1S507 (Ip36.1); D1S436 (Ip36.1); D1S233 (Ip35); D1S201 (Ip35); D1S496 (Ip34.3); D1S197 (Ip1); D1S209 (Ip31.1–p33); D1S216 (Ip31.1–p33); D1S465 (Ip1); D1S207 (Ip31.2–p33); D1S488 (Ip31.3–p33); D1S167 (Ip1.31); D1S435 (Ip1.31); D1S188 (Ip31.3); D1S424 (Ip31.3); D1S236 (Ip31.3); D1S121 (Ip32.4–p22); D1S1067 (Ip31.1–p14.3); D1S1234 (Ip31.1–p14.2); D1S1300 (Ip31.2–p14.2); D1S1233 (Ip31.2–p14.1); D1S127 (Ip31.2–p14.1); D1S1210 (Ip31.4–p12); GLUT2 (Ip32.2–q27); D6S262 (6q21–q22.1); D6S292 (6q22–q23); D6S311 (6q1–q25); D6S290 (6q21–q25.2); ESR (6q21.3); D6S305 (6q25.2–q27); D6S264 (6q25.2–q27); D6S281 (6q27); D1S1922 (11p15.5); D1S1907 (11p13); D1S903 (11p13–q13); D1S925 (11q23.3); D1S912 (11q23.5–q25); D1S924 (11q24–q25); D1S117 (13q2); D1S1246 (13); D1S260 (13q12.3); D1S171 (13q13.2–q13); D1S1695 (13q12–q13); D1S1694 (13q12–q13); D1S267 (13q22.3); D1S219 (13q23–q23.3); D1S263 (13q14.1–q14.2); D1S1693 (13q14.1–q14.3); D1S176 (13q14.3); D1S1690 (13q13.2–q22); D1S154 (13q13.3–q32); D1S173 (13q32–q34); D1S285 (13q34); D1S6265 (16q21); D1S6422 (16q24.2); D1S6413 (16q24.3); D1S6305 (16q24.3); TP53 (17p13.1); THRA (17q11.2–q12); D17S800 (17); D17S855 (17q21); D17S801 (17); D17S802 (17); D17S784 (17); D17S192 (17); D20S186 (20); D20S191 (20); D20S190 (20); D20S192 (20); D20S120 (20q13.1–q13.2); and D20S171 (20q13.2–q13.3). Twenty-five ng of DNA samples were subjected to PCR analysis in a total volume of 25 µl. DynaZyme polymerase (Finzymes Oy, Espoo, Finland) was used in the buffer solution provided by the manufacturer with 100 µM of each deoxynucleotide triphosphate and 0.25 µM primers. After 5 min denaturation at 94°C, samples

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3 The abbreviations used are: LOH, loss of heterozygosity; CGH, comparative genomic hybridization.
were subjected to 35 cycles of amplification consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension for 10 min at 72°C. The PCR products were denatured in formamide buffer and separated on 6.5% polyacrylamide sequencing gels and transferred to a Hybond-N+ nylon membrane (Amersham, Aylesbury, UK). Hybridization and visualization of the PCR products were based on a peroxidase labeled probe (ECL kit; Amersham) as described by Vignal et al. (20) with minor modifications (21). LOH was inspected visually by comparing the intensity of alleles from normal and tumor DNA. Absence or significant decrease in the intensity of one allele relative to the other was considered as an allelic imbalance.

**Statistical Analysis.** The χ² and Fisher’s exact tests were used to compare differences between the frequency of genetic aberrations observed in the BRCA2 999del15 group and frequencies observed in the control group.

**RESULTS**

In general, tumors from BRCA2 999del15 carriers showed a higher frequency of LOH than in controls. The frequency of LOH in cases varied from 47% (11p and 20q) to 92% (13q; Table 1). LOH detected at chromosomes 3p, 6q, 11p, 11q, 13q, and 17p was significantly higher in tumors from the carriers than in the control group (Table 1).

The results of LOH analysis determined with individual markers at these chromosomes are shown in Fig. 1, as well as the P obtained from the statistical analysis.

The highest frequency of LOH at the 3p region in the BRCA2 999del15 tumors is detected by the D3S1210 marker (63%) and is significantly higher than in the control group (P < 0.001; Fig. 1A). This marker is located at the 3p12–p14.1 region. A significant difference was also detected with markers D3S1217 (located at 3p14.1–p14.2), D3S1067 (located at 3p14.3–p21.1), and D3S1300 and D3S1234 (located at 3p14.2–p21.1 within the FHIT gene). In tumors where both D3S1234 and D3S1300 were informative, a consistency of LOH was detected in most cases. Both markers showed LOH in 16 tumors (62%), both markers showed retention of heterozygosity in 8 tumors (31%). LOH with marker D3S1234 but not with marker D3S1300 was detected in two tumors (8%), and LOH with marker D3S1300 and retention of heterozygosity with marker D3S1234 was not detected in any tumor. In contrast, no significant difference was seen in LOH frequency between the groups detected by the marker GLUT2, mapping to 3q (Fig. 1A).

The results for chromosome 6q are shown in Fig. 1B. The frequency of LOH is significantly higher in the carriers at all markers tested, except for D6S262, the most centromeric marker. The majority of cases showing LOH have lost all or a large part of chromosome 6q, and a minority of cases show loss at a small region telomeric to marker D6S1703 (Fig. 2A). At chromosome 11 two markers, i.e., D11S922 mapping to 11p and D11S925 mapping to 11q, show a significant difference between the two groups (Fig. 1C). The markers (D11S907 and D11S903) mapping near the centromere detect a low LOH frequency in both groups (Fig. 1C). Microdeletions are detected at both arms of chromosome 11, separately in individual tumors or

<table>
<thead>
<tr>
<th>Chromosome arm</th>
<th>Sporadic tumors</th>
<th>Tumors in 999del15 carriers</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>1p</td>
<td>89/207 (43%)</td>
<td>21/41 (51%)</td>
<td>0.33</td>
</tr>
<tr>
<td>3p</td>
<td>57/200 (29%)</td>
<td>25/38 (66%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6q</td>
<td>52/141 (37%)</td>
<td>27/36 (754%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>11p</td>
<td>16/78 (21%; Ref. 17)</td>
<td>16/34 (47%)</td>
<td>0.005b</td>
</tr>
<tr>
<td>11q</td>
<td>33/99 (33%; Ref. 17)</td>
<td>24/36 (67%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>13q</td>
<td>71/139 (51%; Ref. 18)</td>
<td>36/59 (62%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>16q</td>
<td>10/151 (60%; Ref. 19)</td>
<td>17/28 (61%)</td>
<td>0.44</td>
</tr>
<tr>
<td>17p</td>
<td>31/75 (41%)</td>
<td>28/32 (88%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>17q</td>
<td>53/90 (59%)</td>
<td>29/40 (73%)</td>
<td>0.14</td>
</tr>
<tr>
<td>20q</td>
<td>43/132 (33%)</td>
<td>21/46 (47%)</td>
<td>0.11</td>
</tr>
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* 99.9% confidence interval.
  b 99.5% confidence interval.
Furthermore, 21% of tumors showed elevation of chromosome 13qLOH, with group 2 consistent with loss at 11p, group 3 consistent with loss at 11q, group 4 shows a complex LOH at 11q, and group 5 shows a complex LOH involving both arms of chromosome 11. In all tumors, group 1 is consistent with total loss of chromosome 11, group 2 is consistent with loss at 11p, group 3 is consistent with loss at 11q, group 4 shows a complex LOH at 11q, and group 5 shows a complex LOH involving both arms of chromosome 11. In C, group 1 is consistent with total loss of 13q, group 2 is consistent with deletions of various sizes at chromosome 13q, and group 3 shows a complex LOH at chromosome 13q. 2, LOH; O, heterozygosity retained; @, homozygosity.

A trend toward larger aberration in the tumors of the BRCA2 999del5 carriers was noted. In tumors showing LOH at chromosome 6q, a major loss of large chromosome region was detected in 27% of sporadic tumors and in 68% of BRCA2 999del5 tumors (P = 0.001). Twenty-five % of sporadic tumors with aberration at chromosome 11 show LOH at markers from both 11p and 11q, whereas 44% of chromosome 11 LOH-positive BRCA2 999del5 tumors show aberrations at both chromosome arms (not a statistically significant difference). Of tumors showing LOH at 13q, 21% in the sporadic group and 27% of the BRCA2 999del5 group showed total loss of chromosome 13 (not statistically significant). Furthermore, 5% of sporadic tumors and 82% of BRCA2 tumors with 13qLOH showed large deletion between markers D13S283 and D13S176 (Fig. 2C), a region of 44 cM (P < 0.001).

**DISCUSSION**

In this study, we report an elevation of LOH at several chromosome arms in tumors of BRCA2 999del5 carriers compared with tumors from individuals without this mutation. All of these regions have been implicated previously in sporadic breast cancer, but in most cases at lower frequency.

Elevation of 3p LOH in BRCA2 999del5 carriers is in line with our earlier finding in a study of sister pairs with breast cancer carrying the germ-line mutation (23). The affected region harbors several known genes, e.g., BAP1, located on chromosome 3p21.3. It has been shown recently to function in the BRCA1 growth-control pathway, and its protein product binds to the RING finger domain of the BRCA1 protein (24). Another gene of interest is the FHIT gene at 3p21.1–p14.2. Several reports have described abnormalities in the FHIT gene in breast carcinomas (25–27). Furthermore, the FHIT gene has been shown to suppress tumorigenicity of cancer cells (28). The FHIT gene encompasses the carcinogen-sensitive common fragile site, FRA3B (29). Another but less studied fragile site of potential interest with respect to the chromosome regions analyzed in this study is FRA6E at chromosome 6q26.

Earlier findings based on the loss of wild-type chromosome 13q suggested a strong selection of tumor cells with both alleles of the BRCA2 gene being mutated (6–7). Here we have shown that the loss at 13q involves the BRCA2 gene in the majority of tumors. The mutation status of the BRCA2 gene at the wild-type chromosome in the three tumors with retention at the BRCA2 locus is unknown, but somatic BRCA2 mutations are considered to be rarely found in breast tumors (30, 31). A highly significant elevation of LOH is detected in cases at all markers between D13S217 and D13S176. It includes
marker D3S153, located close to the BR1 tumor suppressor gene. This suggests that there might not only be an enhanced growth selection toward the loss of the BRCA2 gene but also for the RB1 gene or another tumor suppressor gene located at 13q.

The region at chromosome 17 that shows an significant elevation of LOH in BRCA2 999del5 tumors in comparison to sporadic tumors harbors the TP53 gene (Fig. 1E; Ref. 22). Knockout mouse experiments have suggested that p53 protein is accumulating in BRCA2-defective mice and that a cell cycle checkpoint mechanism is activated due to defective BRCA2 protein and corresponding DNA damage (32). It has been suggested that accumulation of p53 protein can reduce the malignant behavior of BRCA2-defective tumors due to cell cycle checkpoint activation (33). In only a minority of BRCA2 999del5 tumors showing LOH at the TP53 locus, TP53 mutation is detected in the remaining allele (22). A growth advantage of BRCA2-defective tumors may be enhanced if only one copy of the TP53 gene is deleted or the LOH target is another gene in the close vicinity of TP53.

The association of both BRCA1 and BRCA2 proteins with the RAD51 protein establishes a direct link between these proteins and the control of genomic integrity and stability because Rad51 is required for meiotic and mitotic recombination events and the repair of double-stranded DNA breaks (34, 35). Furthermore, fibroblasts from BRCA2 knockout mice show defects in DNA repair of double-stranded breaks (33). Defects in homologous recombination result in numerous spontaneous chromosomal abnormalities in fibroblasts from BRCA2 knockout mice (36). Our results may reflect the inability of mutated BRCA2 to participate in the Rad51-mediated repair. A putative model of LOH selection might be that due to improper fidelity in mitotic recombination and corresponding DNA repair; the BRCA2 999del5 follows a more aggressive pathway of chromosome damage than tumors without this mutation, but in most cases the same chromosome regions are involved in both groups. Whether loss of additional genes is involved in the tumor progression of BRCA2 999del5 carriers compared with sporadic tumors remains unsolved.

In this study, we used microsatellite markers to detect somatic changes in breast tumors. The results from our study support the results of a CGH study comparing tumors from BRCA2 carriers with sporadic breast tumors, with a few exceptions (13). We detected a significant elevation of LOH at the 17p region, not detected in the CGH study, and although a general elevation of allelic imbalance in BRCA2 tumors versus sporadic ones is detected, the difference is not as high as according to CGH. This slight discrepancy might be explained by the different resolution power of the two methods. CGH is not sensitive to allelic losses that are caused by mechanisms other than large physical deletions. Indeed, the architecture of the LOH is different in the two tumor groups at some chromosome regions, because the BRCA2 999del5 tumors show that larger regions are involved. In tumors showing loss at a given chromosome arm, specific regions can be preferably affected in the BRCA2 999del5 tumors in comparison with sporadic tumors. Our findings possibly pinpoint candidate loci for the search for genes that when inactivated promote tumor progression in individuals predisposed to breast cancer due to the BRCA2 999del5 mutation.

The large number of abnormal chromosomes observed in tumor cells in BRCA2 999del5 individuals supports the belief that BRCA2 is involved in maintaining appropriate chromosome segregation and/or chromosomal repair. The high number of genetic defects that are detected in BRCA2 carriers indicates that germline mutation of the BRCA2 gene results in an accelerated accumulation of secondary somatic genetic changes in the tumors. This acceleration could explain the aggressive phenotype of tumor growth in tumors from BRCA2 999del5 carriers, because breast cancer associated with the BRCA2 999del5 mutation are high grade tumors with a rapid proliferation rate (37).

The chromosome defects described in this study are likely to be helpful in the understanding of the somatic genetic progression pathways that contribute to the development of malignancy in genetically predisposed individuals.

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