Allelic loss at chromosome 13q12–q13 is associated with poor prognosis in familial and sporadic breast cancer

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Summary Loss of heterozygosity (LOH) was analysed in 84 primary tumours from sporadic, familial and hereditary breast cancer using five microsatellite markers spanning the chromosomal region 13q12–q13 which harbours the BRCA2 breast cancer susceptibility gene, and using one other marker located within the RB1 tumour-suppressor gene at 13q14. LOH at the BRCA2 region was found in 34% and at RB1 in 27% of the tumours. Selective LOH at BRCA2 occurred in 7% of the tumours, whereas selective LOH at RB1 was observed in another 7%. Moreover, a few tumours demonstrated a restricted deletion pattern, suggesting the presence of additional tumour-suppressor genes both proximal and distal of BRCA2. LOH at BRCA2 was significantly correlated to high S-phase values, low oestrogen and progesterone receptor content and DNA non-diploidy. LOH at BRCA2 was also associated, albeit non-significantly, with large tumour size and the ductal and medullar histological types. No correlation was found with lymph node status, patient age or a family history of breast cancer. A highly significant and independent correlation existed between LOH at BRCA2 and early recurrence and death. LOH at RB1 was not associated with the above mentioned factors or prognosis. The present study does not provide conclusive evidence that BRCA2 is the sole target for deletions at 13q12–q13 in breast tumours. However, the results suggest that inactivation of one or several tumour-suppressor genes in the 13q12–q13 region confer a strong tumour growth potential and poor prognosis in both familial and sporadic breast cancer.

Keywords breast cancer; BRCA2; retinoblastoma gene; allelic loss; prognosis

The study of hereditary cancer syndromes and identification of mutated predisposing genes has provided clues to the initial genetic events in carcinogenesis, some of which may also be involved in sporadic forms of the diseases (Knudsen, 1993). This model may also be used in elucidating the multifactorial cause of breast cancer, a disease in which genetic components have been implicated but in which precursor lesions are hard to define and analyse.

A major breast and ovarian cancer susceptibility gene, BRCA1 on chromosome 17q21, has been identified by positional cloning (Miki et al., 1994). The frequent finding of putative loss-of-function germline mutations in breast/ovarian cancer families and the loss of the wildtype allele in corresponding tumours suggests that BRCA1 is a tumour-suppressor gene inactivated by classical mechanisms. However, although loss of heterozygosity (LOH) of the 17q21 region and a reduced BRCA1 expression is observed in invasive sporadic breast cancer (Thompson et al., 1995), somatic BRCA1 mutations are infrequent in breast and ovarian tumours (Futreal et al., 1994; Merajver et al., 1995), suggesting that the role of BRCA1 in tumorigenesis may be restricted to the hereditary form of the disease.

A second major breast cancer susceptibility gene, BRCA2, was recently identified at chromosome 13q12, proximal to the retinoblastoma (RB1) gene at 13q14 (Wooster et al., 1995; Tavtigian et al., 1996). The findings of frequent LOH at chromosome 13q in breast cancer (Varley et al., 1989) have previously been assumed to be owing to the involvement of the RB1 gene in tumour development. However, in an earlier study (Borg et al., 1992), we found an incomplete correlation between LOH and loss of RB1 expression, suggesting that other adjacent genes might also be targets for deletions. This presumption was subsequently reinforced by the isolation of Bruch-1, a gene proximal to RB1 on 13q12–q13 which manifests reduced expression in tumours with LOH (Schott et al., 1994). Thus, three or more potential tumour-suppressor genes may reside in the 13q12–q14 region, supporting the theory that a complexity of gene rearrangement exists in tumours. The present study was undertaken to analyse the LOH in microsatellite markers flanking the BRCA2 locus and its correlation to prognostic factors in tumours from both familial and sporadic breast cancer.

Materials and methods

Patients and tumours

Eighty-four patients diagnosed for primary breast cancer in the age range 26–80 years were included. Cases were included into one of three categories according to patient family history of cancer, as obtained from clinical records and patient interviews: (1) Hereditary breast cancer (n = 30), three or more first- or second-degree relatives (including the index case) with breast or ovarian cancer, at least one of which had an early age (<50 years) of onset; (2) Familial breast cancer (n = 16), two first- or second-degree relatives (including the index case) with breast or ovarian cancer, at least one before the age of 50 or, alternatively, three or more cases over the age of 50; (3) Sporadic breast cancer (n = 39), no cancer of any kind in first- or second-degree relatives of the index case. Data on tumour size, lymph node status, histological type and clinical follow-up were obtained from patient records. A subset of the familial and hereditary cases included in the present study has been investigated for BRCA1 and BRCA2 germline mutations and/or linkage (Johannsson et al., 1996; Håkansson et al., submitted).

Tumours were analysed for oestrogen (ER) and progesterone (PgR) receptors (PgR) with enzyme immunoassays and for DNA ploidy and S-phase fraction with DNA flow cytometry, according to previously described protocols (Fernsö et al., 1992). ERBB2 and 11q13 (INT2) amplifications were assessed by slot blot analysis on extracted tumour DNA (Borg et al., 1991).

PCR microsatellite analysis

The polymerase chain reaction (PCR) was used to detect allelic imbalance (designated here as LOH) at polymorphic microsatellite markers by comparing the allelic pattern in

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tumour and blood DNA. Six chromosome 13q markers were analysed using primers with published sequence (Gyapay et al., 1994) purchased from Research Genetics (Huntsville, AL, USA). The markers were, from centromere to telomere, D13S290, D13S260, D13S267, D13S219, D13S263 and D13S153. The first three markers reside within the 6 cm BRCA2 region at chromosome 13q12-q13 as initially defined (Wooster et al., 1994), while D13S153 lies within the RB1 gene at 13q14. The same patient material was also analysed for LOH at markers on chromosome 16q. The markers were, from telomere to centromere, D16S261, S16S308, D16S186, D16S301, D17S138, D16S305 and D16S303 (Research Genetics). The PCR mixture (30 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 1.1 – 2.5 mM magnesium chloride, 0.13 µM of each primer, 20 µM of dNTPs, 0.75 units of Taq polymerase (Boeringer Mannheim) and 80 ng of genomic DNA. The forward primer had been radiolabelled with T4 polynucleotide kinase (Promega) and [32P]ATP (> 5000 Ci mmol–1, Amersham). The PCR was carried out in an OmniGene thermocycler (Hybaid) and consisted of one cycle of 4 min at 93°C, followed by 28 – 32 cycles of 1 min at 93°C, 1 min at 52-68°C, 1 min at 72°C, followed by one cycle of 5 min at 72°C. An aliquot of 1 – 8 µl PCR product was mixed with denaturing loading buffer (95% deionised formamide, 10 mM sodium hydroxide, 0.05% bromophenol blue, 0.05% xylene cyanol), heated for 5 min at 95°C, cooled on ice and loaded (4 µl) on 0.4 mm thick, preheated and denaturing (8 M urea) 6% polyacrylamide gels for electrophoresis at 45 – 50°C for 4 h. The gels were transferred to chromatography paper, and autoradiographed (by exposing radiographs for 10 – 72 h at –70°C). In order to accurately compare the band intensities, care was taken to use approximately equal concentrations of PCR products within each analysed tumour/blood pair.

Statistical analysis
The correlation between dichotomised variables were compared by chi-square and Pearson analysis. Differences in survival between subgroups of patients were compared with the log-rank test, and the survival curves were computed according to the method of Kaplan and Meier. Multivariate survival analysis was done according to the Cox proportional hazard model. All computations were executed with the Stata software (Stata Corporation, Release 3.1, 6th edition, College Station, TX, USA).

Results
Frequency and pattern of LOH
Forty percent of all 84 tumours manifested LOH for at least one informative marker on chromosome 13q. Most often these alterations involved both the BRCA2 (D13S290, D13S260 and D13S267) and the RB1 (D13S153) loci. LOH at the BRCA2 region was found in 34% of 83 informative tumours and at RB1 in 27% of 60 informative tumours. Six tumours manifested LOH limited to markers within the BRCA2 region, whereas LOH at RB1 was found in another six tumours with retained heterozygosity at BRCA2. A striking feature of the deletions found on chromosome 13q was that the majority exhibited the near complete loss of one allele, compared with the often more partial allelic loss of markers from chromosome 16q (data not shown).

A few tumours manifested a restricted pattern of LOH within the BRCA2 region (Figure 1). In tumour no.6723 from a hereditary breast cancer, clear LOH was evident at D13S260 but not at D13S219 (D13S267 and D13S153 being uninformative), in keeping with the more selective LOH in the BRCA2 region. Similarly, in tumour no.7928 from a sporadic breast cancer, LOH was manifest at D13S267, but not at D13S219 or D13S153 (D13S260 being uninformative). However, in tumour no.8649 from a hereditary breast cancer, LOH was observed at D13S290 but not at D13S260, D13S267 or D13S153, suggesting that a gene proximal to BRCA2 may be involved. Moreover, in tumour no.6814 from a familial breast cancer (Figure 1), LOH was found at D13S219 and D13S263, but not for markers at the...
BRCA2 or RBI loci, suggesting the presence of an additional target gene distal to D13S267 and BRCA2 but proximal to RBI.

Correlation to other clinical and tumour characteristics

A strongly significant correlation was found between LOH at BRCA2 (i.e. LOH at one or more of D13S290, D13S260 and D13S267) and a high rate of proliferation (S-phase fraction, SPF). More than 80% of the most rapidly proliferating tumours manifested LOH at BRCA2, compared with merely 8% within the group of slowly growing tumours (Table 1). The mean SPF in the group showing LOH was 12.5% (median 12%), whereas the corresponding percentage in the group without LOH was as low as 5.8 (median 5.1%). A similar trend, but without statistical significance, was found between LOH at RBI and the level of S-phase fraction. A significant relationship was also found between LOH at BRCA2 and lack of ER and PgR expression. Moreover, allelic loss at BRCA2, but not at RBI, was associated with DNA non-diploidy. However, no correlation was found to LOH at chromosome 16q or to amplification of the ERBB2 gene or the chromosomal region 11q13. Ductal tumours manifested a slightly higher frequency of LOH at both BRCA2 and RBI, compared with lobular tumours, although the number of lobular tumours was too low for reliable comparison to be made. Interestingly, LOH at BRCA2 was observed in four of five medullar tumours, three of which were informative at RBI and manifested retained heterozygosity.

There was no difference in the frequency of LOH at BRCA2 or RBI in tumours from patients with either sporadic, familial or hereditary disease, nor in respect of different age groups (Table 1). A strong association between LOH at BRCA2 and high SPF values was observed in all three groups of breast cancer, mean SPF values in tumours manifesting LOH being 13.2% (sporadic) 18.5% (familial) and 11.2% (hereditary), compared with 5.2%, 5.7% and 6.1%, respectively, in the groups of tumours with retained heterozygosity at BRCA2.

Finally, while there was a similar high frequency of LOH in node-negative and node-positive tumours, a trend was seen towards a higher rate of LOH at BRCA2 in tumours of larger size.

Correlation to prognosis

A strongly significant correlation was found between LOH at BRCA2 and a shortened recurrence-free survival (Figure 2); a significant relationship being found also to overall survival.

Table 1  Relationship between loss of heterozygosity at BRCA2 or RBI, and other clinicopathological and biological/genetic factors in breast cancer

| Variable | BRCA2 n LOH % P-value | RBI n LOH % P-value |
|----------|------------------------|----------------------|
| Total    | 83 34 60 27            |                      |
| Type of disease |               |                      |
| Sporadic | 38 34 27 26           |                      |
| Familial | 16 31 11 36           |                      |
| Hereditary | 29 34 NS 22 23       | NS                   |
| Age      |                       |                      |
| < 40 years | 19 32 13 15          |                      |
| 40–50 years | 36 33 30 27       |                      |
| ≥ 50 years | 28 36 NS 17 35      | NS                   |
| Node status |                   |                      |
| 0        | 39 36 31 19           |                      |
| 1–3      | 24 29 19 37           |                      |
| > 3      | 16 38 NS 6 33         | NS                   |
| Tumour size |                   |                      |
| < 20 mm  | 51 27 42 29           |                      |
| > 20     | 31 45 NS 17 24        | NS                   |
| Histology type |               |                      |
| Ductal   | 67 30 48 31           |                      |
| Lobular | 6 17 5 20             |                      |
| Medular  | 5 80 3 0              |                      |
| Miscellaneous | 5 60 0.056 4 0     | NS                   |
| ER       |                       |                      |
| < 25 fmol mg⁻¹ | 33 48 21 24   |                      |
| ≥ 25     | 50 24 0.021 39 28    | NS                   |
| PgR      |                       |                      |
| < 25 fmol mg⁻¹ | 39 46 23 35   |                      |
| ≥ 25     | 44 23 0.024 37 22   | NS                   |
| DNA ploidy |                   |                      |
| Diploid  | 22 18 18 17           |                      |
| Non-diploid | 30 47 0.033 19 26 | NS                   |
| S-phase fraction |         |                      |
| < 7%     | 24 8.3 20 10          |                      |
| 7–12%    | 12 50 7 29            |                      |
| ≥12%     | 11 82 < 0.001 8 50   | 0.060                |
| ERBB2    |                       |                      |
| Single copy | 31 39 21 33       |                      |
| Amplified | 4 50 NS 3 33         | NS                   |
| 11q13    |                       |                      |
| Single copy | 31 45 21 33       |                      |
| Amplified | 3 33 NS 3 33         | NS                   |
| 16q     |                       |                      |
| No LOH   | 23 22 17 35           |                      |
| LOH      | 36 42 NS 25 28        | NS                   |

*Combined data from markers: D16S261, D16S308, D16S186, D16S301, D16S318, D16S305 and D16S303. NS, non-significant.

Figure 2  Disease-free survival in 83 breast cancers categorised after LOH at the BRCA2 locus (a) and in 60 breast cancers categorised after LOH at the RBI gene (b).
Allelic deletion and loss of heterozygosity constitutes the second event in the genetic two-hit model, according to which both copies of a tumour-suppressor gene are inactivated (Knudsen et al., 1993). Consequently, the findings of a high frequency of LOH at the BRCA1 breast cancer susceptibility gene on chromosome 17q21 both in hereditary and sporadic breast tumours are strongly suggestive of a general involvement of this putative tumour-suppressor gene in breast tumorigenesis. However, while the unmasking of a recessive BRCA1 mutation by deletion of the remaining wildtype allele holds to be true in hereditary BRCA1-linked tumours (Smith et al., 1992; Johannsson et al., 1996), the evidence for the importance of BRCA1 in sporadic breast cancer with LOH on 17q21 is still controversial (Futreal et al., 1994).

The objective of the present study was to investigate the involvement of the chromosomal region 13q12–q14, comprising the BRCA2 and RB1 loci, and its possible clinical importance in breast cancer. In accordance with the findings at BRCA1, a similarly high frequency (> 30%) of LOH was found at BRCA2 in sporadic, familial and hereditary tumours. Moreover, not only was the frequency of LOH lower at RB1, but the relationship to aggressive tumour phenotype (high proliferation, autonomous growth, genetic instability, etc.) and poor prognosis was more or less confined to tumours manifesting allelic loss at BRCA2, with or without concomitant loss of RB1. This implies that BRCA2, or an additional adjacent gene other than RB1, is the likely target for 13q deletions in breast cancer, in keeping with our earlier observations of a lack of association between LOH at RB1 and loss of pRB expression (Borg et al., 1992).

Strong evidence for the inactivation of BRCA2 via the postulated two-hit mechanism was recently provided in studies of breast cancer and other tumours from disease haplotype carriers of a BRCA2-linked family, demonstrating a preferential loss of the wild type allele (Collins et al., 1995; Gudmundsson et al., 1995). In the present study, information on BRCA2 linkage and/or germline mutations was available only for a subset of familial and hereditary cases. In one of the hereditary cases included (Lund 11), a single basepair (G) deletion at nucleotide 4486 in exon 11 of BRCA2 has been identified (Håkansson et al., submitted), creating a premature termination at codon 1447. The tumour from Lund 11 (no.8648) did, indeed, manifest LOH at BRCA2 and also at RB1. In 12 of the remaining 36 familial and hereditary cases of the present study, screening of all exons in BRCA2 was performed, giving no further evidence of germline mutations. Only in 2 of these 12 cases was LOH at the BRCA2 locus present. Germline BRCA1 mutations have previously been described (Johannsson et al., 1996) in three of the cases included in the present study (Lund 33, 44 and 56); a fourth case manifested clear BRCA2 linkage (Lund 1). In two of these four cases LOH was present in all informative 13q markers, whereas one case manifested retained heterozygosity at the BRCA2 locus. Interestingly, in the fourth case (no.6814 from Lund 33), LOH was seen at D13S219 and D13S263 but not at the BRCA2 and RB1 loci, implying the existence of an additional gene of importance in the 13q12–q14 region, telomeric of BRCA2 and centromeric of RB1 (Figure 1). Restricted LOH at a region centromeric of BRCA2 was also observed, suggesting the presence of other putative tumour suppressor genes. Obviously, 'Brush-1' may be one such gene as it is affected by deletion and reduced expression in tumours without alterations at RB1 (Schoot et al., 1994). The presence of at least three tumour-suppressor genes in the 13q12–q14 region may explain why extensive deletions, or even the loss of a whole chromosome 13, are common and selected for in breast cancer (Devilee et al., 1989). One previous investigation of sporadic breast cancer and alterations in the 13q12–q14 region demonstrated allelic loss in 32% of 200 tumours and a simultaneous loss of both the BRCA2 and RB1 loci in all cases (Cleton-Jansen et al., 1995), whereas another report has pointed out the restricted involvement of the BRCA2 loci in some tumours (Kerangueven et al., 1995).

The finding of the near complete loss of one allele (merely a faint band visible, presumably representing DNA from non-invasive tissue within the tumour) in a considerable proportion of tumours suggests that 13q deletion is an early step in tumour development. Additionally, loss of a 13q12–q14 gene may confer a strong growth advantage to the cell, resulting in a selective outgrowth of cell clones which harbour the losses. Furthermore, the strong correlation between LOH and high S-phase fraction values indicates the inactivation of a gene (or several genes) involved in cell cycle control. It is unlikely that these associations are due to a general genomic instability, as there was no correlation between LOH at chromosome 13q and 16q, and as the latter alteration was unrelated to prognosis. However, the present study provides no proof that the association with aggressive tumour behaviour is specifically as a result of inactivation of the BRCA2 gene. Certainly, in a parallel study of biological tumour features from individuals with germline BRCA1 mutations or manifesting clear BRCA1 linkage, a relationship between aggressive phenotype and rapid proliferation has been noted (Johannsson et al., submitted). This implies that a functional similarity between these breast cancer susceptibility genes may exist and that their inactivation initiates a dedifferentiated state and a certain genetic pathway, leading to rapid tumour progression. Although we have seen only a tendency towards a worse prognosis in BRCA1-induced
breast cancer (Johannsson et al., submitted), LOH at the chromosomal region comprising the BRCA2 gene was strongly and independently correlated to early recurrence and death. This association was evident in both familial and hereditary tumours as well as in sporadic tumours, suggesting that alterations of the putative target gene constitutes a new prognostic factor of potential clinical importance.

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