Frequent somatic loss of BRCA1 in breast tumours from BRCA2 germ-line mutation carriers and vice versa

S Staff1, JJ Isola1, O Johannsson2, Å Borg2 and MM Tanner1

1Laboratory of Cancer Genetics, Institute of Medical Technology, University Hospital of Tampere, FIN-33014 University of Tampere, Finland; 2Department of Oncology, University Hospital, S-221 85 Lund, Sweden

Summary Breast cancer susceptibility genes BRCA1 and BRCA2 are tumour suppressor genes the alleles of which have to be inactivated before tumour development occurs. Hereditary breast cancers linked to germ-line mutations of BRCA1 and BRCA2 genes almost invariably show allelic imbalance (AI) at the respective loci. BRCA1 and BRCA2 are believed to take part in a common pathway in maintenance of genomic integrity in cells. We carried out AI and fluorescence in situ hybridization (FISH) analyses of BRCA2 in breast tumours from germ-line BRCA1 mutation carriers and vice versa. For comparison, 14 sporadic breast tumours were also studied. 8 of the 11 (73%) informative patients with tumours Ca 7936 and Ca 6 were identified in breast cancer tumours from germ-line BRCA1/2 mutation carriers and vice versa suggests that somatic events occurring at the BRCA1 and BRCA2 loci in sporadic tumours (Staff et al, 2000). Half of the tumours (4/8) showed a physical deletion of the BRCA1 gene by FISH. Combined allelic loss of both BRCA1 and BRCA2 gene was seen in 12 of the 17 (71%) informative hereditary tumours, whereas copy number losses of both BRCA genes was seen in only 4/14 (29%) sporadic control tumours studied by FISH. In conclusion, the high prevalence of AI at BRCA1 in BRCA2 mutation tumours and vice versa suggests that somatic events occurring at the other breast cancer susceptibility gene locus may be selected in the cancer development. The mechanism resulting in AI at these loci seems more complex than a physical deletion. © 2001 Cancer Research Campaign

Keywords: BRCA1; BRCA2; allelic imbalance; LOH; FISH

Approximately 5–10% of breast cancer is due to inherited predisposition (Miki et al, 1994). Germ-line mutations in the two identified susceptibility genes, BRCA1 (Miki et al, 1994) and BRCA2 (Wooster et al, 1994; Tavtigian et al, 1996) are responsible for a large proportion of hereditary breast cancer (Szabo and King, 1997). Both BRCA1 and BRCA2 are considered as classical tumour suppressor genes and therefore inactivation of both alleles is required for cancer initiation. Although no sequence homology has been found between BRCA1 and BRCA2, they share many functional properties (reviewed in Welch et al, 2000).

Almost all the tumours from germ-line BRCA1 and BRCA2 mutation carriers show loss of heterozygosity (LOH) or AI at the corresponding loci (Smith et al, 1992; Neuhausen and Marshall, 1994; Collins et al, 1995; Gudmundsson et al, 1995; Staff et al, 2000), which is in accordance with the lost tumour suppressor function. Due to several functional parallels between BRCA1 and BRCA2, we studied the possible somatic aberrations of BRCA1 by AI and FISH in breast cancer tumours from germ-line BRCA2 mutation carriers, and vice versa. The possible concomitant somatic aberrations of the BRCA1 and BRCA2 genes were also studied in 14 sporadic breast cancer samples by FISH. We have previously shown (Staff et al, 2000) that unlike in hereditary BRCA1/2 tumours, the allelic imbalance at BRCA1/2 loci is almost always a result of a physical deletion in sporadic tumours. Therefore, physical deletion of the BRCA1 and BRCA2 genes detected by FISH reflects the allelic imbalance of the BRCA1/2 loci in sporadic tumours (Staff et al, 2000).

MATERIALS AND METHODS

Patients and tumour samples

17 primary breast cancer tumours from germ-line BRCA1 mutation carriers and 8 primary breast tumours from germ-line BRCA2 mutation carriers were derived from the Department of Oncology, University of Lund. 14 primary sporadic breast cancer tumours were obtained from Tampere University Hospital. The tumour samples were snap-frozen and stored at −70°C until used for AI and FISH analyses.

Genomic DNA was extracted from available blood samples of the 13 BRCA1 and 6 BRCA2 germ-line mutation carriers by standard methods. One BRCA1 patient had 2 separate tumours (Ca 8571 and Ca 13996; Table 1), which were both analysed. BRCA1 patients with tumours Ca 14090 and Ca 14007 (Table 1) were relatives, but none of the other BRCA1 patients were directly related. One BRCA2 patient had also 2 separate tumours (Ca 11900 and 14 486; Table 2). BRCA2 patients with tumours Ca 7936 and Ca 11506 were from the same family, similarly as patients with tumours Ca 11787 and Ca 13816 (Table 2). BRCA1 and BRCA2 mutation analyses have been described previously (Johansson et al, 1996; Hákansson et al, 1997; Tables 1 and 2).

PCR microsatellite analysis

Polymerase chain reaction (PCR) was used to detect AI at polymorphic microsatellite markers by comparing the allelic patterns...
Table 1  Copy number aberrations of BRCA2 by FISH and AI in 17 breast cancers from germ-line BRCA1 mutation carriers

| Tumour | BRCA1 mutation | Result of the BRCA1 mutation | AI at the BRCA1 locusa | AI at the BRCA2 locusb | DNA Indexc | Mean copy number/cell of BRCA2 (±SEM) | Mean copy number/cell of 13q reference probe (±SEM) | Mean copy number ratio (BRCA2/13q reference probe) | Interpretation of the BRCA2 copy number by FISHd |
|--------|----------------|-----------------------------|------------------------|------------------------|-----------|-------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Ca 12 421 | 2594delC | Ile845Stop | NA | NA | 1.56 | 3.84 (0.11) | 2.55 (0.09) | 1.51 | 3.4 BRCA2 gain |
| Ca 11 808 | 3829delT | Leu1263Stop | Yes | Yes | 1.0 | 1.18 (0.09) | 1.20 (0.09) | 0.98 | Monosomy of 13q |
| Ca 09 252 | 2594delC | Ile845Stop | Yes | Yes | 1.8 | 2.47 (0.11) | 3.45 (0.10) | 0.72 | 3.2 BRCA2 deletion |
| Ca 14 007 | 3172ins5 | Thr1025Stop | Yes | Yes | 1.53 | 1.43 (0.08) | 2.71 (0.10) | 0.53 | 3.2 BRCA2 deletion |
| Ca 10 581 | 1806C→T | Gin563Stop | Yes | No | 1.73 | 2.17 (0.12) | 3.66 (0.16) | 0.59 | 4.2 BRCA2 deletion |
| Ca 12 224 | 1806C→T | Gin563Stop | Yes | Yes | 1.52 | 2.10 (0.10) | 2.50 (0.11) | 0.84 | Large deletion at 13q |
| Ca 14 510 | 300T→G | Cys61Gly | Yes | Yes | 2.46 | 2.43 (0.17) | 2.53 (0.15) | 0.96 | Large 13q deletion |
| Ca 10 360 | 3172ins5 | Thr1025Stop | Yes | Yes | 1.7 | 1.80 (0.09) | 1.94 (0.07) | 0.98 | Large 13q deletion |
| Ca 13 812 | 4808C→G | Glu1115Stop | Yes | No | 1.87 | 1.82 (0.08) | 1.98 (0.08) | 0.92 | Large 13q deletion |
| Ca 13 714 | 5382insC | Glu1829Stop | Yes | No | 2.48 | 3.30 (0.18) | 3.06 (0.09) | 1.08 | Large 13q deletion |
| Ca 13 996 | 1806C→T | Gin563Stop | Yes | Yes | 1.11 | 1.84 (0.09) | 2.24 (0.10) | 0.82 | No relative copy number change |
| Ca 14 970 | 2594delC | Ile845Stop | Yes | No | 1.00 | 2.28 (0.11) | 2.21 (0.09) | 1.02 | No relative copy number change |
| Ca 11 394 | 1177G→A | Trp353Stop | NA | NA | 1.0 | 2.40 (0.14) | 2.95 (0.14) | 0.81 | No relative copy number change |
| Ca 08 822 | 1201del11 | Ser361Stop | Yes | No | 1.89 | 2.27 (0.15) | 5.44 (0.27) | 0.42 | 5:2 BRCA2 deletion |
| Ca 10 697 | Linkage ++ | | | | | | | | |
| Ca 14 090 | 3172ins5 | Thr1025Stop | Yes | No | 1.00 | 2.08 (0.10) | 2.19 (0.08) | 0.95 | No relative copy number change |
| Ca 08 571 | 1806C→T | Gin563Stop | Yes | No | 3.20 | 3.19 (0.19) | 3.05 (0.18) | 0.96 | No relative copy number change |

Copy numbers represent the mean of at least 50 nuclei counted from each sample. (NA = Not available, NI = Not informative) *Previously published (Staff et al., 2000). Allelic imbalance was analysed using microsatellite markers 13S267 and 13S260. AI was stated if at least one of the markers used indicated imbalance (compared to normal DNA) of more than 25% between the alleles in tumour sample. DNA index by DNA flow cytometry. Deletion was defined if the copy number ratio was 0.80 or less. Gain was defined if the copy number ratio was 1.30 or more. *3:2 BRCA2 deletion in a subpopulation. When DNA-index was used as copy number reference, the copy number ratios indicated a large deletion in 13q spanning both BRCA2 and ETB genes. When 13q probe (ETB) was used as a reference probe, no BRCA2 gene copy number change was revealed.

Table 2  Copy number aberrations of BRCA1 by FISH and AI in 8 breast cancers from germ-line BRCA2 mutation carriers

| Tumour | BRCA2 mutation | Result of the BRCA2 mutation | AI at the BRCA1 locusa | AI at the BRCA2 locusb | DNA Indexc | Mean copy number/cell of BRCA1 (±SEM) | Mean copy number/cell of chr 17 centromere (±SEM) | Mean copy number ratio (BRCA1/chr 17 cen) | Interpretation of the BRCA1 copy number by FISHd |
|--------|----------------|-----------------------------|------------------------|------------------------|-----------|-------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Ca 11 900 | 2024del5 | Ser599Stop | Yes | Yes | 1.89 | 2.27 (0.15) | 5.44 (0.27) | 0.42 | 5:2 BRCA1 deletion |
| Ca 10 588 | 4486delG | Val1447Stop | NA | NA | 1.07 | 1.10 (0.04) | 2.0 (0.07) | 0.55 | 2:1 BRCA1 deletion |
| Ca 13 816 | 3058A→T | Lys944Stop | Yes | Yes | 1.00 | 1.18 (0.05) | 4.0 (0.00) | 1.18 | Monosomy of chromosome 17 |
| Ca 14 486 | 2024del5 | Ser599Stop | Yes | Yes | 1.87 | 2.14 (0.11) | 4.42 (0.20) | 0.48 | 4:2 BRCA1 deletion |
| Ca 07 936 | 6293C→G | Ser2022Stop | No | Yes | 1.00 | 2.19 (0.13) | 3.24 (0.14) | 0.94 | No relative copy number change |
| Ca 11 721 | 5445del5 | Tyr1739Stop | NA | NA | 1.00 | 3.04 (0.18) | 3.68 (0.13) | 0.83 | No relative copy number change |
| Ca 11 787 | 3058A→T | Lys944Stop | Yes | Yes | 1.94 | 4.08 (0.12) | 3.92 (0.09) | 1.04 | No relative copy number change |
| Ca 11 506 | 6293C→G | Ser2022Stop | Yes | No | 1.96 | 2.77 (0.12) | 2.27 (0.07) | 1.22 | No relative copy number change |

Copy numbers represent the mean of at least 50 nuclei counted from each sample. (NA = Not available). Allelic imbalance was analysed using microsatellite markers 13S267 and 13S260. AI was stated if at least one of the markers used indicated imbalance (compared to normal DNA) of more than 25% between the alleles in tumour sample. DNA index by DNA flow cytometry. Deletion was defined if the copy number ratio was 0.80 or less. Gain was defined if the copy number ratio was 1.30 or more.
of tumour and blood DNA. Two *BRCA1* intragenic markers (D17S855 and D17S1322) (Albertsen et al, 1994) and 2 markers physically linked to *BRCA2* (D13S260 and D13S267) (Wooster et al, 1994) were analysed using primers with published sequence (Gyapay et al, 1994) (Research Genetics, Huntsville, AL, USA). The PCR reactions were carried out as previously described (Staff et al, 2000). 1 µl of the PCR product was analysed by capillary electrophoresis using ABI PRISM™310 Genetic Analyser and GeneScan 2.1 Software according to the manufacturer’s instructions (Perkin-Elmer). For the informative heterozygous markers, the AI was determined by calculating ratio of the alleles (L) as previously described (Staff et al, 2000). If L < 0.75 or L > 1.33, then one of the alleles has decreased more than 25% resulting in AI, as previously defined (Keranguen et al, 1997).

**FISH analyses**

FISH analyses were performed using gene-specific PAC probes for *BRCA1* (PAC 103014) and *BRCA2* (PAC 92M18) genes. The specificity of these clones has previously been confirmed (Staff et al, 2000). Chromosome 17 centromere probe (p17H8) was used as a copy number reference for *BRCA1*. For *BRCA2*, a PAC probe specific for the *ETB* gene (at 13q22) was used as a reference, because specific centromere probe for chromosome 13 is not available. The hybridization efficiency of the probes was tested in a non-malignant breast sample. Hybridization and detection were performed as previously described (Tanner et al, 1998; Staff et al, 2000). Hybridization signals from 50–100 nuclei were scored to assess the copy number of the *BRCA1* and *BRCA2* genes. Deletion of the *BRCA* genes was defined as an average ratio ≤ 0.80 of *BRCA1*/*2* signals relative to chromosome 17 centromere signals or *ETB* signals, respectively. Gain was defined as an average ratio of ≥ 1.30. Digital images were taken with a Hamamatsu 9585 camera (Hamamatsu, Hamamatsu City, Japan) operated via ISIS image analysis software (MetaSystems, Altlussheim, Germany).

**RESULTS**

**BRCA1 and BRCA2 tumours**

11 out of 13 *BRCA1* mutation carriers with available blood samples were informative, i.e. they were heterozygous for at least one of the two *BRCA2* markers. AI at *BRCA2* was found in 8 of the 11 (73%) informative cases (Figure 1, Table 1). All the 17 *BRCA1* tumours were analysed for the *BRCA2* gene copy number by FISH. 3 tumours showed a clear physical interstitial deletion of *BRCA2* gene when *BRCA2* signals were compared to the reference gene signal counts (*ETB* gene at 13q22) (Figure 1, Table 1). If the overall ploidy level (= DNA index by flow cytometry) was used as a *BRCA2* copy number reference, 6 additional tumours showed a loss of *BRCA2*. This suggests a large deletion at 13q comprising both *ETB* and *BRCA2* genes in all but one of these tumours.

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*Figure 1* Examples of the assessment of allelic imbalance (AI) by automated DNA sequencer and two-colour FISH of *BRCA1*, chromosome 17 centromere, *BRCA2* and *ETB* (13q reference probe). The AI and FISH analyses of the same tumour are presented next to each other so that AI analysis is shown in the left. The fragment analysis of PCR products is shown from tumour DNA (top rows) and from matched blood DNA (bottom rows). Size of PCR products (in base pairs) is shown on the X-axis, and the peak heights in fluorescence units are shown on the Y-axis. The alleles in the normal DNA and the corresponding peaks in the tumour DNA are shown in grey. The corresponding allele peak areas in informative tumours are presented in boxes next to the peaks. In FISH images, the probes are visualised in green and red colours (fluorescein and Texas Red, respectively). The probes are marked with the corresponding colour in each panel. The nuclei were counterstained with DAPI (blue). The case numbers are marked in each panel with white colour texture. (A) Tumour 11 900 from germ-line *BRCA2* mutation carrier showing AI at the *BRCA1* locus with marker D17S855 and physical deletion of *BRCA1* by FISH. (B) Tumour 11 787 from germ-line *BRCA2* mutation carrier demonstrating AI at the *BRCA1* locus with marker D17S855 and no relative *BRCA1* gene copy number change by FISH. (C) Tumour 09 252 from germ-line *BRCA1* mutation carrier showing AI at the *BRCA2* locus with marker D13S260 and physical deletion of *BRCA2* by FISH. (D) Tumour 10 360 from germ-line *BRCA1* mutation carrier showing AI at the *BRCA2* locus with marker D13S260 and no *BRCA2* gene copy number change relative to 13q reference probe by FISH.
copy number loss was present in 4 (29%) out of 14 genes by FISH only in 4 (29%) out of 14 tumours. One tumour (1/17; 6%) showed a copy number loss of the BRCA2 gene, which is detectable by FISH.

The concomitant loss of BRCA2-linked breast cancers, which showed combined LOH at the 17q21 locus (Figure 1, Table 1). 7 out of 17 (41%) of the sporadic tumours showed loss of both BRCA2 copy number change, yet 2 of these cases showed AI of BRCA2 copy number change, yet 2 of these cases showed AI of BRCA2 copy number change and 3:2 BRCA2 deletion, respectively (Nagai et al, 1991). In Ca 12 224, ETB copy number loss was present in 53% (9/17) of the BRCA2 gene copy number change.

All but one of the informative BRCA1 tumours showing change in the relative BRCA2 gene copy number showed also AI at the BRCA1 locus (Figure 1, Table 1). 7 out of 17 (41%) of the BRCA1 tumours did not reveal any relative BRCA2 copy number change, although 2 of them (i.e. 2 out of 4 informative cases) showed AI of BRCA2 (Table 1). One tumour (1/17; 6%) showed a copy number gain of the BRCA2 gene but this tumour was not available for AI analysis (Table 1).

5 of the available 6 BRCA2 tumours (83%) showed AI at the BRCA1 locus (Figure 1, Table 1). All the tumours were also analysed by FISH, and 4 of them (4/8; 50%) showed a physical deletion of the BRCA1 gene (Figure 1, Table 2). All the informative cases with deletion of AI at the BRCA1 locus (Figure 1, Table 2). 4 of 8 (50%) tumours revealed no relative BRCA1 copy number change, yet 2 of these cases showed AI of BRCA1 (Figure 1, Table 2).

Sporadic breast tumours

14 unselected primary sporadic breast cancers were analysed for both BRCA1 and BRCA2 gene copy number changes by FISH. Physical deletion of BRCA1 was detected in 6 cases (6/14, 43%). Loss of BRCA2 was present in 7 cases (7/14, 50%). The concomitant deletion of both the BRCA genes was detected by FISH in only 4 tumour samples (4/14, 29%). FISH data of the sporadic tumours are summarised in Table 3.

DISCUSSION

In the present study, we have studied BRCA1 copy number changes and AI in BRCA2 mutation tumours and vice versa. Only one study has been published previously on concomitant allelic loss of BRCA1 and BRCA2 in hereditary breast cancer. It involved 7 BRCA1-linked breast cancers, which showed combined LOH at BRCA1/2 loci at high level (Kelsell et al, 1996). Unfortunately, due to low incidence of BRCA mutation tumours, studies of BRCA1/2 tumour features have been complicated by small sample size. Nevertheless, we were here able to study a reasonable number of BRCA1 cases and extend the study to concern also BRCA2 tumours. Our results showed a high prevalence (73% in BRCA1 tumours, Table 1:67% in BRCA2 tumours, Table 2) of combined AI of BRCA genes in both BRCA1/2 tumours.

Taken together both BRCA1 and BRCA2 tumours available for AI analyses, concomitant allelic loss were detected in 12 (71%) out of 17 cases. In contrast, the set of sporadic breast cancer showed loss of both BRCA genes by FISH only in 4 (29%) out of 14 tumours (Table 3). We have shown previously that AI of both BRCA genes in sporadic breast cancer results mainly from physical deletion of the BRCA genes, which is detectable by FISH. Therefore, we think that it is possible to compare hereditary AI data with FISH data from sporadic tumours. When the frequency of concomitant loss of BRCA1/2 genes was statistically compared between hereditary and sporadic tumours, a significant difference between these two groups was found (Pearson $\chi^2 = 5.43; P < 0.02$). Sporadic breast cancers reported in literature also have shown combined LOH of BRCA1 and BRCA2 at lower frequency (47% in Kelsell et al, 1996; 32% in Silva et al, 1999) than in the hereditary tumours analysed here. In sporadic cancers, LOH/AI has frequently been seen at either BRCA1 or BRCA2 locus, at 17q21 (24–38%) or 13q12–13 (18–63%), respectively (Nagai et al, 1994; Hamann et al, 1996; van den Berg et al, 1996; Niederacher et al, 1997; Phelan et al, 1998). However, controversy exists whether AI/LOH at the single BRCA locus is clinically significant in sporadic tumours (Beckmann et al, 1996, Bieche et al, 1997; Silva et al, 1999).

Our results imply that combined AI at the BRCA loci might reflect a common pathway in tumour progression of hereditary breast cancers. In contrast, BRCA1/2 were concomitantly affected only in a minority of sporadic breast cancers, which further suggests that concomitant somatic loss of BRCA genes is a typical feature of hereditary and not sporadic breast tumours.

Comparison of FISH and AI data makes it possible to distinguish whether allelic imbalance is due to a physical deletion or whether it is due to other genetic mechanisms. In general, BRCA

| Sporadic cases | Interpretation of BRCA1 copy number by FISH* | Interpretation of BRCA2 copy number by FISH* |
|---------------|---------------------------------------------|---------------------------------------------|
| Case 1**      | 4:2 BRCA1 deletion                          | 3:2 BRCA2 deletion                          |
| Case 2**      | 4:2 BRCA1 deletion                          | 3:2 BRCA2 deletion                          |
| Case 3        | No relative BRCA1 copy number change        | Monosomy of chromosome 17                   |
| Case 4        | No relative BRCA1 copy number change        | No relative BRCA2 copy number change        |
| Case 5**      | Monosomy of chromosome 17                   | Monosomy of chromosome 13                   |
| Case 6        | No relative BRCA1 copy number change        | 3:2 BRCA2 deletion                          |
| Case 7**      | 4:2 BRCA1 deletion                          | 3:2 BRCA2 deletion                          |
| Case 8        | No relative BRCA1 copy number change        | 3:2 BRCA2 deletion                          |
| Case 9        | No relative BRCA1 copy number change        | No relative BRCA2 copy number change        |
| Case 10       | 2:1 BRCA1 deletion                          | No relative BRCA2 copy number change        |
| Case 11       | No relative BRCA1 copy number change        | No relative BRCA2 copy number change        |
| Case 12       | 2:1 BRCA1 deletion                          | No relative BRCA2 copy number change        |
| Case 13       | No relative BRCA1 copy number change        | No relative BRCA2 copy number change        |
| Case 14       | No relative BRCA1 copy number change        | No relative BRCA2 copy number change        |

*Deletion was defined if the copy number ratio (BRCA1 gene copy number signals/chromosome 17 centromere signals or BRCA2 gene copy number signals/ETB gene copy number signals) was 0.80 or less. **Concomitant loss of BRCA1 and BRCA2.
copy number changes and AI were in good agreement. However, in some cases AI was detected in the absence of actual gene copy number loss suggesting that deletion does not always explain AI. In theory, illegitimate homologous mitotic recombination could promote AI without any actual gene copy number losses, which are detected by FISH. However, whether these findings are truly linked to BRCA mutation tumours, requires further studies.

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