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Original Paper

Chromosome Imbalance at the 3p14 Region in Human Breast Tumours: High Frequency in Patients with Inherited Predisposition due to *BRCA2*

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Our previous studies have indicated that genetic aberrations in the 3p14 region are more frequent in malignant tumours from hereditary breast cancer patients than sporadic breast cancers. The main purpose of this study was to test if *BRCA2* susceptibility alleles contribute to imbalance in the 3p14 region. We mapped allelic imbalance at 3p14 in tumours from Icelandic sisters affected with breast cancer using a set of 10 microsatellite markers (tel-*D3S1295-D3S1234-D3S1300-D3S1600-D3S1233-D3S1217-D3S1261-D3S1296-D3S1210-D3S1284*-cen). The patients were of known carrier status with respect to the 999del5 mutation in *BRCA2* which is the most common cause of hereditary breast cancer in Iceland. Of 103 patients, 32 in the group were mutation carriers. A high degree of imbalance was observed in tumours from *BRCA2* mutation carriers, ranging from 44 to 88% for individual markers. This was significantly higher than the percentage of imbalance in tumours from non-carriers, where the frequency ranged from 25 to 43%. In both groups, we noted elevated 3p14 imbalance in patients with bilateral disease. Allelic imbalance was most commonly observed near the marker *D3S1210* (3p14.1–p12) and the *FHIT* gene (3p21.1–p14.2) for both groups. We conclude that genomic aberrations in 3p14 are especially frequent in tumours with *BRCA2* gene defects, and suggest that this is caused by regional loss of chromosome stability rather than selection. © 1998 Elsevier Science Ltd.

Key words: breast cancer, allelic imbalance, chromosome 3p14, *BRCA2* carriers

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INTRODUCTION

Loss of genetic material from the 3p region is frequently observed in several types of malignancies including breast, lung, oesophagus and renal cancer [1–4]. In a study on breast tumours, Chen and associates [5] identified two regions on 3p, at 3p14–p13 and 3p26–p24, that appear to be independently lost in breast tumours suggesting the existence of two different tumour suppressor genes in the region important for breast tumorigenesis. Cytogenetic studies have indicated that 3p deletions appear early in tumour formation since they are found in premalignant stages such as fibroadenomas and proliferative breast disease [6]. In addition, one of the most

frequently detected genetic lesions observed in near diploid breast tumours is a deletion affecting the proximal region of this chromosome arm [7].

An important landmark in the 3p region is the commonly expressed fragile site FRA3B, located at 3p14.2. Spontaneous chromosomal breaks in cultured cells are notably induced in this region by the drug aphidicholin [8, 9]. FRA3B maps near the breakpoint of a constitutional translocation t(3;8) that predisposes to renal cancer [10]. Both the t(3;8) translocation breakpoint and FRA3B are located within a region of the *FHIT* gene. *FHIT* is a member of the histidine triad gene family and encodes for an enzyme with ‘dinucleoside 5′,5′′′-P1,P3-triphosphate hydrolase’ activity [11]. Genomic rearrangements affecting *FHIT* are found in gastrointestinal, breast, lung and Merkel cell carcinoma [12–16]. The protein

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tyrosine phosphatase γ receptor gene (*PTPR γ*) mapping closely centromeric to *FHIT* is a second tumour suppressor gene candidate in the 3p14 region [17]. Homozygous deletions involving both genes have been reported in proliferative breast disease [18].

We have previously mapped allelic imbalance in the 3p region in sporadic breast tumours and tumours derived from hereditary breast cancer patients [19, 20]. Comparison of the two patient groups revealed differences, mainly accounted for by excessive imbalance at more proximal loci (3p14) in tumours from hereditary breast cancer patients. The hereditary breast cancer subjects in this study belonged to families that showed positive linkage to the *BRCA2* locus, and shared a common disease haplotype [21]. The causative *BRCA2* allele was later characterised as a 5 base pair deletion in exon 9, termed 999del5 [22, 23]. This allele was detected in 8.5% of Icelandic breast cancer patients [24].

In the present study, we examined the relationship between 3p14 instability in tumours and the 999del5 genotype by mapping allelic imbalance (AI) in breast tumour samples from a group of sisters affected with breast cancer that had been screened for the presence of the 999del5 mutation.

MATERIALS AND METHODS

The study group consisted of sisters diagnosed with breast cancer before the age of 60 years. Pedigree data and information concerning disease status was obtained from the Icelandic Genetic Council, Icelandic Cancer Registry and Department of Pathology at the University Hospital of Iceland. A total of 109 tumours from 103 individuals were available for the study. The patient group had been screened for the 999del5 mutation and the associated haplotype (A. Arason, University Hospital of Iceland, Iceland). 32 individuals in this series had the 999del5 germline mutation. The group was also screened for the only *BRCA1* mutation (G5193A) known to occur in the Icelandic population [25]. 2 of the patients were positive for this mutation. In addition, a previous study based on a combination of linkage and loss of

heterozygosity (LOH) analysis [26] indicated that these were the only *BRCA1* and *BRCA2* mutations affecting the group (A. Arason, University Hospital of Iceland, Iceland).

The mean age of diagnosis was 48.8 years for the control group and 46.5 for 999del5 carriers. All but two of the samples were from primary tumours. Histologically, two samples were classified as medullary, three as tubulo-ductal and three as non-invasive intraductal cancer. The rest were classified as ductal invasive cancer. All tumours from 999del5 carriers were ductal invasive cancers. The distribution of family sizes in the study group was as follows: 5 families had 3 members, 34 of the families had 2 members and 20 families were represented by a single patient. In the 999del5 group, these figures were 2, 10 and 6, respectively.

Paraffin-embedded normal and malignant tissue from the patients was obtained for analysis from the Department of Pathology at the University Hospital of Iceland. In the case of malignant tissue, areas rich in tumour cells (>90%) were selected for analysis by microscopy. Tissue slices (20 μ m) were incubated at 55°C for 24 h in a solution containing protease, Tween 20 and Nonidet P-40 [27]. This lysate was boiled for 10 min and cooled on ice prior to application in polymerase chain reactions (PCR).

The markers were chosen on the basis of their location and success with archival material. Information on the markers (Table 1), cytogenetic locations and genetic distances were obtained from GDB (Genomic Database, Johns Hopkins School of Medicine, Baltimore, Maryland, U.S.A.).

Thermal cycling was typically carried out in 30 μ l volumes containing: 0.3 units Dynazyme polymerase (Finnzymes Oy, Espoo, Finland), the reaction buffer provided with the enzyme, 100 μ M each dNTP, 0.5 μ l DNA sample, 50 ng of each primer. Cycling conditions were as follows: 94°C for 5 min; 35 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 40 s. The samples were mixed with formamide loading buffer and electrophoresed in a conventional sequencing gel (6% acrylamide, 7 M urea). The amplified products were transferred by contact blotting on to a N+Hybond (Amersham

Table 1. The percentage of allelic imbalance (AI) at each locus and percentage of breaks between marker loci in the combined sister group is shown here. The cytogenetic location of the markers is also shown and the approximate genetic distance between markers in centimorgans, calculated from Genome Database maps

Marker	Cytogenetic location	Distance (cM)	Total AI percentage (number informative)	Total breaks percentage (number informative)
D3S1295	3p21.1-14.2	3.2	34 (44)	20 (15)
D3S1234	3p21.1-14.2	0.6	57 (30)	32 (22)
D3S1300	3p21.1-14.2	4.9	47 (59)	30 (43)
D3S1600	3p14.2-14.1	3.5	51 (68)	14 (37)
D3S1233	3p14.2-14.1	4.1	47 (66)	14 (43)
D3S1217	3p14.2-14.1	5.3	49 (73)	20 (46)
D3S1261	3p14.1-p12	0.0	41 (64)	21 (19)
D3S1296	3p14.1-p12	1.6	48 (31)	12 (16)
D3S1210	3p14.1-p12	4.0	54 (67)	9 (42)
D3S1284	3p14.1-p12		52 (64)	

International, Buckinghamshire, U.K.) nylon membrane and fixed with ultraviolet illumination. Either one of the amplification primers was used to probe the amplified product. The primer was elongated with terminal transferase and covalently

bound to peroxidase. After hybridisation with extended primers, the membranes were washed, bathed in luminol and exposed to X-ray films. Elongation of oligonucleotides, hybridisation and visualisation of the PCR products were as described by Barkardottir and associates [28], using the ECL system (Amersham International). Two of the markers were additionally typed with a detection method based on incorporation of radiation end-labelled ($[\gamma^{32}\text{P}]\text{ATP}$) amplification prior to thermal cycling reactions (see Figure 1). Allelic imbalance was estimated by visual comparison of tumour and normal band intensities by at least two viewers.

Statistical calculations: Chi square, Fishers exact, T- and F-statistics were performed with aid of the StatView program package (Abacus Concepts, Inc., Berkeley, California, U.S.A.).

RESULTS

The frequency of AI at individual markers ranged from 34 to 57% in the whole study group (Table 1). The highest frequency in the 999del5 group was found at the *D3S1296* locus (88%) and the second most frequent target was at the adjacent locus, *D3S1210* (80%). In the control group, the highest frequency of AI was detected with the marker *D3S1210* (43%). The frequency of AI in tumours from 999del5 carriers exceeded that in the control group for every marker tested (Figure 2). The observed difference was significant with markers *D3S1600* ($P=0.0033$), *D3S1217* ($P=0.001$), *D3S1261* ($P=0.0015$), *D3S1210* ($P=0.007$) and *D3S1284* ($P=0.0112$). Of all informative typings from the 999del5 group, 70% ($n=195$) scored as AI compared with 37% of the typings on control material ($n=371$). In order to compare regional differences between *BRCA2* mutation carriers and the control group, we pooled the results with respect to the

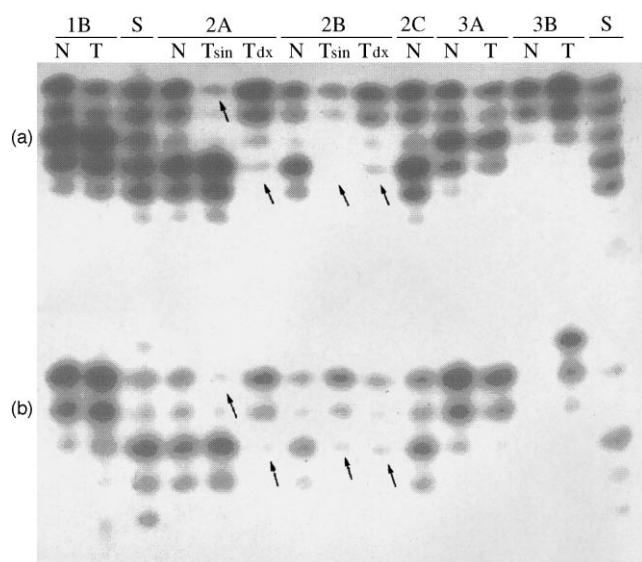


Figure 1. Representative autoradiogram showing the genotype for markers *D3S1217* (a) and *D3S1210* (b) in samples from normal (N) and tumour (T) tissue. The standards used (S) were mixtures of reactions that gave a ladder suitable for sample comparison. The arrows point to tumour alleles with diminished intensity. Individuals 2A, 2B, 2C are sisters with an identical genotype for the two markers. Imbalance is seen with both markers in all four tumours of the bilateral breast cancer patients, 2A and 2B.

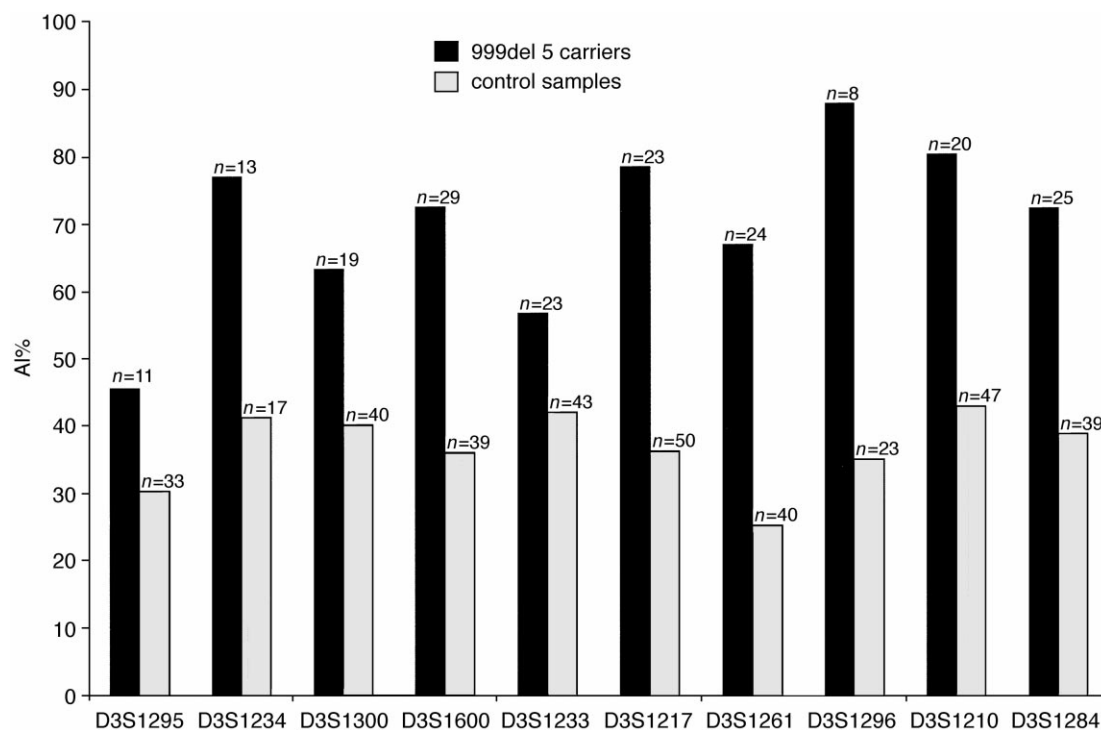


Figure 2. Percentage of breast tumours showing allelic imbalance with the markers used in the study. The two groups under comparison are: affected sisters with the 999del5 mutation in *BRCA2* (solid bars) and the control group of affected sisters (shaded bars) negative for the mutation. Breast cancer was diagnosed before the age of 60 years in all subjects in the study. The number of informative tests (n) for each marker is shown above the bars.

three cytogenetically defined regions. AI within a given region was scored positive whenever one or more of the included markers showed imbalance. The greatest difference between groups was in the 3p14.1–p12 region ($P=0.0009$, $n=105$). Significance was also apparent for the pooled data from 3p14.2–p14.1 ($P=0.0079$, $n=105$) but less so with 3p21.1–p14.2 markers ($P=0.0162$, $n=69$).

Maps for tumours with partial allelic imbalance are shown in Figure 3. Interstitial imbalance involving only one marker is not common, but was evident in two cases at the *D3S1300* locus (tumours 4 and 5 in Figure 3). Smallest regional overlap (SRO) analysis indicated that two regions are preferably involved in allelic imbalance. These are bordered by *D3S1300* and *D3S1600* in the distal part and by *D3S1210* and *D3S1296* in the more proximal region.

Although, according to our statistical comparison, there was a preference for imbalance at proximal loci in the 999del5 positive tumours, this was not reflected in the imbalance maps. We did, however, note a trend towards larger aberrations in the tumours from this group. To test this further, we compared the groups with respect to the frequency of 'total' imbalance i.e. imbalance affecting the whole region. Here, we only considered samples with five or more informative markers in order to reduce the number of tumour genomes that would be falsely taken to have imbalance in the whole region. Using these criteria, samples with imbalance stretching over the whole 3p14 region were significantly ($P=0.0026$) more frequent among 999del5 carriers than controls. Additionally, comparison was made with respect to tumours displaying partial imbalance in the region (AI seen with some but not all markers). Here, AI was significantly more dense in the 999del5 group ($P=0.0071$).

The observation of imbalance and lack of imbalance detected with two adjacent markers in a single tumour may be referred to as chromosome breaks. The number of breaks were too few to allow comparison of regional distribution. However, we counted breaks in the combined group in order to compare their distribution with the known location of aphidicholin-induced breaks. The most frequent spot for such breaks is seen in the *D3S1300* region (see Table 1) and there is also an additional frequency peak with respect to breaks in the region *D3S1261*.

A total of 17 tumours from 11 patients with bilateral breast cancer were analysed in this study. AI with at least one marker was observed in 16 of these tumours. The tumours were non-randomly distributed between the groups since 11 of the 17 were from 999del5 carriers ($P=0.005$, by Fisher's exact test). To examine this further, we compared the percentage of imbalance between groups that are categorised both according to 999del5 status and manifestation of bilateral disease. When a comparison was made with all available typings, allelic imbalance was most frequent (88%, $n=60$) in tumours from 999del5 carriers with bilateral disease, and 61% ($n=135$) in the group of 999del5 carriers with unilateral disease. The prevalence in non-carriers with bilateral disease was 55% ($n=31$), and 35% ($n=340$) in the control group of unilateral cancer patients.

DISCUSSION

The results presented here indicate that allelic instability in the 3p14 region is more frequent in carriers of the 999del5 mutation than in a control group with negative *BRCA2* mutation status. Although our methodology does not allow discrimination between allelic loss and gain, we assume, in accordance with previous publications, that the majority of detected imbalance is caused by deletions [5,29]. In our opinion, the observed difference between the groups can be explained in two ways: Cooperative selection may influence tumour development, and accordingly, selection for loss of genes in the 3p14 region is stronger in the absence of *BRCA2*; or loss of *BRCA2* function may affect 3p14 stability in the tumours.

The difference between imbalance frequencies in the groups is more significant for the 3p14.1–p12 region. Although this is consistent with a 'cooperative' locus in the region, the observation can be biased as markers from the distal (3p21.1–p14.1) region have fewer informative typings. The fact that tumours from *BRCA2* mutation carriers are affected by an imbalance covering larger portions of the 3p14 region implies failure in the mechanism maintaining chromosome stability. The observed effect could be generated by interaction with genomic structures, e.g. aphidicholin-sensitive fragile sites that are found in the region [9]. Increased expression of fragile sites in lymphocytes from breast cancer

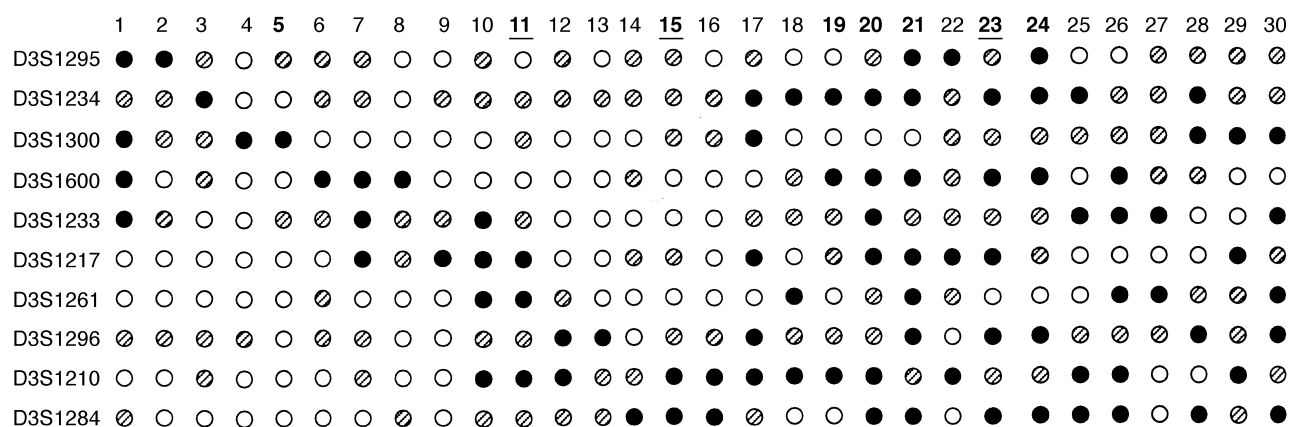


Figure 3. Patterns of allelic imbalance (AI) at 3p14 in human breast tumours from affected sister-pairs. Solid circles denote AI, open circles represent intact regions and cross-hatched circles denote uninformative typings. Only tumours that have partial AI and are informative with five or more markers are shown here. Tumours from 999del5 carriers are identified with a bold number (cases 5,11,15,19,20,21,23,24) and tumours from bilateral cancer patients have underlined numbers (cases 11,15,23).

patients supports this view [30,31]. The region around marker *D3S1300* stands out as the one most commonly involved in terms of breaks (Table 1). This locus maps intragenic to the *FHIT* gene and adjacent to *FRA3B* [12]. Breaks are also common around the *D3S1261* locus in the tumours, a region containing fragile sites [9] that are expressed at a higher concentration of aphidicholin (4 μ M) than *FRA3B*.

Tumours from bilateral breast cancer patients appear particularly prone to 3p14 aberrations. Age at diagnosis and distribution of histological subtypes were the same in these patients as the rest of the group. Our observation suggests that the molecular development of these tumours is different from that in unilateral breast cancer, in line with the conclusion of Kinoshita and associates [32] who found high incidence of *p53* mutations in bilateral breast cancers.

Although high frequency of imbalance at 3p14 in *BRCA2*-deficient tumours may reflect a higher degree of genomic instability, the resulting genetic changes are likely to be favoured by selection. From the smallest regional overlaps, we conclude that tumour suppressor genes contributing to selection in the region are most likely to be in the interval between the markers *D3S1210* and *D3S1296*. A second candidate region is bordered by *D3S1300* and *D3S1600* including the first five exons of the *FHIT* gene and the *PTPR γ* gene. Recent findings indicate that *FHIT* is not a selective target for deletions in this region [33].

Assuming the possibility that the 3p loci have an additive impact on the risk of breast cancer in the families studied here because of a segregating tumour suppressor gene, one predicts that the chromosome containing the disease lesion is retained in the tumours affected by LOH. We compared the alleles retaining intensity in tumours from relatives showing allelic imbalance. In our study, the alleles retained in the tumours were not preferably identical. Furthermore, allelic preferences were not noted in the tumour pairs available from bilateral cancer patients as 3 (out of 6) retained different alleles (see Figure 1).

There is increasing evidence for heterogeneity with respect to the molecular evolution of breast cancer. Interestingly, *BRCA1* and *BRCA2* mutations are rarely found in sporadic breast cancers [34–36], suggesting that selection may favour other genes. Recently, a study comparing secondary genetic changes in tumours from *BRCA1* and *BRCA2* carriers to those found in sporadic tumours was performed using the comparative genome hybridisation (CGH) method [37]. The results showed large differences between the three groups with regard to location and frequency of allelic losses and gains. The majority of *BRCA2* defective tumours in the study were derived from 999del5 mutation carriers. In general, there was a higher frequency of allelic loss throughout the genome in the *BRCA2* tumours than the sporadic tumours, the difference reaching statistical significance at 13cen-q21, 11q14–q21, 6q and 3p21–cen. Although some fragile sites are known to reside in these areas, other regions containing fragile sites did not have elevated frequency of imbalance. The second most commonly expressed aphidicholin-sensitive site at 16q23 [38] showed a significantly lower deletion frequency in *BRCA2*-defective tumours than in sporadic tumours [37]. However, direct comparison is not straightforward since there are discrepancies between studies attempting to define fragile sites, some of which can be explained by the use of different methods for chemical

induction. Population (genetic) variation and tissue specificity has also been documented in this context.

Reports on the association of both *BRCA1* and *BRCA2* with the Rad51 protein [39,40] establish a direct link between these proteins and the control of genomic integrity since Rad51 is required for meiotic and mitotic recombinational events and repair of double-stranded DNA breaks in yeast and mammalian cells. Our results may reflect the inability of mutated *BRCA2* to participate in Rad51-mediated repair.

In order to identify the biologically important regions in breast carcinogenesis, it may be necessary to be able to define 'cytogenetic' noise or background. In the case of tumours postulated to have a defect in repair of double-stranded DNA breaks, cytogenetic noise is likely to cluster in regions with a high incidence of spontaneous breaks. Therefore, an assessment of such breaks in the normal breast epithelium could provide us with useful information regarding tumour progression.

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