Relation between deletion of chromosome 1p36 and DNA ploidy in breast carcinoma: an interphase cytogenetic study

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Abstract

Aims—To investigate whether deletion of the 1p36 region of chromosome 1 is independent of DNA ploidy in breast cancer cells.

Methods—Preparations of nuclei from 64 fresh primary breast tumours were studied using dual target fluorescence in situ hybridisation (FISH) combining probes specific for the 1q12 (pUC 1.77) and 1p36 (p1-79) regions of chromosome 1. Signals were counted in 100–300 nuclei and the percentage of cells showing fewer p1-79 than pUC 1.77 signals was measured in each sample. DNA ploidy was investigated by cytofluorimetry in 55 tumour samples.

Results—Chromosome 1 aberrations were detected in 56 samples. There were fewer p1-79 than pUC 1.77 signals in 53 samples. The 1p36 region was deleted in 11 samples in which a single p1-79 signal was detected; seven of these samples were diploid. Abnormalities were found in 17/24 diploid and 30/31 aneuploid tumours.

Conclusions—Chromosome 1 aberrations, including deletion of the 1p36 region, were observed in diploid breast tumours. Deletion of the 1p36 region may be an early event in tumorigenesis. Given the frequency and importance of chromosome 1 aberrations in the biological behaviour of breast tumours, FISH, used in conjunction with cytofluorimetry, may be helpful for determining prognosis in patients with diploid tumours.

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Chromosome 1 abnormalities are a recurrent finding in breast carcinoma: karyotypic analysis has shown that numerical and structural aberrations of this chromosome occur in both primary^{1–6} and metastatic breast cancer.^{7,8} As a consequence of various aberrations, either loss of several short arm regions (1p) or gain of the long arm (1q), an allelic imbalance is generated between the two chromosome arms. Loss of the 1p36 region has been demonstrated in a subset of breast cancers by karyotyping and loss of heterozygosity (LOH) studies.^{9–14} Loss of 1p36 has also been found more often in non-diploid tumours and is an indicator of poor prognosis.^{15,16}

In the present study, we have investigated loss of the 1p36 region of chromosome 1

in 64 human breast cancers using dual target fluorescence in situ hybridisation (FISH), combining probes specific for the 1q12 (pUC 1.77) and the 1p36 (p1-79) regions. This technique, which has already been used to detect chromosome deletions in human cancer,^{18–20} allows one to analyse interphase nuclei in single cells. As the number of 1q12 and 1p36 regions can be counted precisely in each cell, deletion of 1p36 can be assumed to have occurred if there are fewer p1-79 signals than centromeric pUC 1.77 signals. FISH is more sensitive than conventional cytogenetics and LOH analysis, permitting the detection of chromosomal changes even in a limited population of cancer cells.

We have therefore investigated the relation between loss of the 1p36 region and DNA ploidy in order to ascertain whether this chromosomal abnormality is also present in diploid breast tumours. Static cytofluorimetry was used to evaluate DNA content.

Methods

Fresh tumour tissue samples were obtained at surgery from 64 patients. A portion of each tumour was immediately fixed in 4% formalin and embedded in paraffin wax. Sections, 4 µm thick, were cut and stained with haematoxylin and eosin and classified according to criteria recommended by the World Health Organisation (WHO).²¹ Another portion of each tumour was placed immediately in sterile culture medium and used for direct culture after mechanical disaggregation. Colcemid (0.02–0.2 µg/ml) was added two to three hours before harvesting. Cells were treated with 0.075 KCl for 20 minutes at 37°C and washed several times with 3:1 methanol:acetic acid.

Lymphocyte nuclei and metaphases were prepared using conventional cytogenetic techniques after being stimulated with phytohaemagglutinin for 72 hours and treated with Colcemid. These preparations were used as controls in the hybridisation experiments.

Nuclei from control and tumour specimens were spread onto slides one to five days before hybridisation.

PROBES

The repetitive pUC 1.77 DNA probe specific for the 1q12 pericentrometric region²² and the repetitive p1-79 DNA probe for the 1p36 telomeric region²³ were used for FISH. Both probes were labelled by nick-translation according to the manufacturer's instructions (Boehringer,

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Mannheim, Germany); pUC 1.77 was labelled with digoxigenin-11-dUTP and p1-79 with biotin-16-dUTP.

FLUORESCENCE IN SITU HYBRIDISATION

Slides of nuclei were treated with RNase (100 µg/ml in 2 × SSC (pH 7) for one hour at 37°C) and pepsin (100 µg/ml in 0.01 N HCl for 15 minutes at 37°C). After washing in phosphate buffered saline (PBS), the nuclei were post-fixed in 1% formalin in PBS/50 mM MgCl₂ for 10 minutes, washed again, dehydrated in ethanol, and dried in air. Nuclear DNA was denatured by dipping the slides into a solution of 70% deionised formamide in 2 × SSC (pH 7) for three minutes at 70°C. The labelled probes (1–2 ng/µl) were dissolved in the hybridisation mixture (50% deionised formamide, 2 × SSC (pH 7), 1 µg/µl salmon sperm DNA, and 10% dextran sulphate) and denatured by heating (one minute in boiling water). Hybridisation was carried out overnight at 37°C.

WASHING AND DETECTION

The slides were washed for 3 × 5 minutes each in 50% formamide in 2 × SSC (pH 7) at 42°C, 5 × 2 minutes in 2 × SSC at 42°C and once in 4 × SSC/0.05% Tween (pH 7) at 37°C.

After a 30 minutes' incubation in 5% bovine serum albumin in 4 × SSC/0.05% Tween (pH 7) at 37°C, the digoxigenin labelled pUC 1.77 probe was detected by the digoxigenin-rhodamine antibody (Boehringer). Biotin-16-dUTP was detected by fluorescein isothiocyanate (FITC) conjugated avidin and amplified with a biotinylated goat anti-avidin antibody (all from Vector Laboratories, Burlingame, California, USA), followed by a second FITC conjugated avidin step. The slides were mounted in a solution containing 20 mg/ml 1,4-diazabicyclo-[2.2.2]octane (DABCO) (Sigma, St Louis, Missouri, USA) and 0.2 mg/ml 4,6-diamidino-2-phenylindole (DAPI) and visualised using a Leitz microscope (Wetzlar, Germany) equipped with filters for FITC, TRITC (tetra-rhodamine isothiocyanate) and DAPI.

EVALUATION

Normal human lymphocytes were used as controls in the hybridisation experiments; 100–300 nuclei were scored for each slide and evaluated according to the criteria of Hopman *et al.*²⁴ The imbalance in FISH signals corresponding to the 1q12 and the 1p36 regions was evaluated by counting the cells in each sample with fewer p1-79 signals than pUC 1.77 centromeric signals. This nuclear fraction is known as the imbalanced nuclear fraction (INF). We also recorded the predominant cell populations present in each sample. Abnormal cell populations were regarded as significant if their numbers exceeded the cut off levels based on the upper limit (mean + 2 SD) found in the control lymphocytes.

Figure 1 Case 1. Dual target FISH. Two spots corresponding to the pUC 1.77 probe (A) and two spots corresponding to the p1-79 probe (B) are visible in two nuclei. Staining with DAPI (C) (× 400).

DNA CYTOFLUORIMETRY

Tumour nuclei were smeared onto glass slides, fixed in 70% ethanol at 4°C and stained with DAPI in PBS for 15 minutes at 4°C. DNA content was analysed by static cytofluorimetry²⁵ using a high gain photomultiplier operated at 600 V. This was connected to a Microphot FXA epifluorescence microscope (Nikon, Tokyo, Japan). For each sample, after subtracting background, the fluorescence intensity of at least 100 nuclei was determined randomly, allowing histograms to be drawn of the DNA content. Samples were classified as diploid when the DNA index (DI) was 1, tetraploid when the DI was 1.80–2.20, or aneuploid when the DI was 1.00–1.79 or >2.20. Lymphocytes from a healthy donor were used as a diploid control.

Table 1 FISH results and DNA index in eight breast cancer samples with normal numbers of signals per nucleus

Case number	WHO*	pUC 1.77/p1-79†	INF	DNA index
1)	MC	85	10	1 (94)
2)	DLC	77	16.5	1 (90)
3)	DC	81.5	14	1 (84)
4)	DC	80	11	1 (88)
5)	DC	84	11.5	1 (88)
6)	DC	72	15	1 (60) 1.62 (30)
7)	DC	72.5	11	1 (82)
8)	DLC	69	17	1 (88)

* Histological diagnosis according to the WHO classification: MC = mucinous carcinoma; DC = ductal carcinoma; DCIS = ductal carcinoma in situ; DLC = ductal/lobular carcinoma; R = recurrence.

† Percentage of cells showing two spots for pUC 1.77 and p1-79.

Table 2 FISH results and DNA index in 22 breast cancer samples with abnormalities and loss of the 1p36 region

Case number	WHO classification	Abnormal cell populations	INF	DNA index
9)	DLC	1* 2/1†	20	1 (92)
10)	R	1 2/1	32	—
11)	DC	1 2/1	29	—
12)	DC	1 2/1	36.5	1 (88)
13)	DC	1 2/1	94.5	1 (92)
14)	MC	1 3/2	14.5	—
15)	DC	1 3/2	16	—
16)	DC	1 3/2	46	1 (72)
17)	DLC	1 3/2	38	1 (80)
18)	MC	1 3/2	60.5	—
19)	LC	1 3/2	71.5	1 (77)
20)	DLC	1 3/2	73.5	—
21)	LC	2 2/1, 3/1	71	1 (85)
22)	LC	2 3/2, 2/1	61.5	1 (88)
23)	DC	2 3/2, 2/1	41	1 (90)
24)	LC	2 3/2, 3/1	78.5	1 (94)
25)	DLC	2 3/3, 3/2	15.5	1 (19) 1.57 (23)
26)	DLC	2 3/2, 3/3	33	—
27)	DC	2 3/3, 3/2	29	1 (17) 1.50 (53)
28)	MC	2 3/2, 3/3	47.5	1 (60) 1.57 (31)
29)	DC	2 3/2, 3/4	27.5	1 (12) 2.06 (71)
30)	DLC	3 3/3, 3/3, 2/1	46	1 (10) 1.9 (74)

* Number of significant abnormal cell populations.

† Signal pattern (pUC 1.77/p1-79).

Results

Of the 64 breast carcinomas investigated, 44 were invasive ductal carcinoma, 10 were invasive ductal/lobular carcinoma, four were invasive lobular carcinoma, four were mucinous carcinoma, one was ductal carcinoma in situ, and one was a recurrence.

CONTROLS

Two large pUC 1.77 signals, corresponding to the pericentromeric regions, were visible in both metaphase and interphase cells (mean (SD) 93.4 (2.9)). The mean percentage of nuclei with one and three spots was 5.9 (3.5) and 0.65 (0.6), respectively. The p1-79 spots were generally smaller in size. In several metaphases, because of replicated DNA, each signal appeared in a paired arrangement (sister chromatids; split spots). Therefore, both in control and tumour cells, paired p1-79 spots were counted as one. The mean percentage of cells with one, two and three spots was 4.6 (2.3), 93.9 (2.8) and 1.25 (1), respectively. Two spots for both probes were counted in 88.3 (3.5) nuclei.

TUMOUR SAMPLES

In eight (12.5%) samples, two spots were observed in 69–85% of nuclei for both probes (fig 1). No other significant cell population was detected in these specimens (table 1).

Figure 2 Case 16. Two and three signals for the pUC 1.77 probe (A) and two signals for the p1-79 probe (B) are visible in two nuclei. Staining with DAPI (C) ($\times 400$).

An abnormal number of signals per nucleus was found in 56 (87.5%) cases, suggesting that chromosome 1 aberrations were present. Of these, 53 had fewer p1-79 than pUC 1.77 signals in a significant percentage of nuclei. The number of spots in these samples varied greatly; samples were divided into two groups based on the number of pUC 1.77 signals.

In group 1 (table 2), a single p1-79 signal was detected in 10.5–83.5% nuclei in 10 samples. It was the only abnormal cell population found in five tumours (cases 9–13). Three 1q12 regions and two 1p36 regions (fig 2) were found as a single abnormal population in seven samples (cases 14–20). Several cell populations were counted in a further 10 samples (cases 21–30): in five of these three spots were detected in 10–19.5% of cells for both probes. A cell population with more p1-79 than pUC

Figures 3 and 4 Cases 36 (fig 3) and 54 (fig 4). Under-representation of the p1-79 probe can be seen clearly figs 3B and 4B, which show two and three p1-79 signals, respectively. Four and five pUC 1.77 signals can be seen in figs 3A and 4A. Staining with DAPI (3C and 4C) ($\times 800$).

1.77 signals was found in one specimen (case 29).

The second group of tumours (group 2) (table 3) was very heterogeneous: the number of pUC 1.77 signals ranged from two to seven, and four 1q12 regions were seen in at least one cell population. The number of p1-79 spots

ranged from two to four in all but one sample (case 31), in which 21% of nuclei had only a single p1-79 spot. Two examples are shown in figs 3 and 4. In these samples we found many cells showing a FISH signal imbalance below 10% which contributed to the high INF values obtained. The INF was significantly higher

Table 3 FISH results and DNA index in 31 breast cancer samples with abnormalities and under-representation of the 1p36 region

Case number	WHO classification	Abnormal cell populations	INF	DNA index
31)	DC	4 3/2, 3/1, 4/2, 2/1	71.5	1 (39) 1.34 (52)
32)	DC	3 3/2, 3/3, 4/2	63	1 (44) 1.79 (41)
33)	DC	2 3/2, 4/2	75	1 (22) 1.43 (49)
34)	DC	2 3/2, 4/2	58.5	1 (4) 1.73 (53)
35)	DC	2 3/2, 4/2	88.5	1 (84)
36)	DC	2 3/2, 4/2	84.5	1.75 (76)
37)	DC	3 3/2, 4/2, 3/3	82.5	1 (65) 1.90 (31)
38)	DC	4 3/3, 4/3, 4/2, 3/2	61	1.58 (86)
39)	DC	4 3/2, 4/3, 3/2, 3/3	69.5	1 (3) 1.86 (75)
40)	DC	4 4/3, 4/2, 3/2, 3/3	76	1 (5) 1.98 (87)
41)	DC	3 4/3, 4/2, 3/3	63.5	1 (24) 1.69 (45)
42)	DC	2 4/3, 3/3	50.5	1 (78)
43)	DC	3 3/2, 3/3, 4/4	37.5	1 (7) 1.93 (60)
44)	DC	5 3/3, 4/3, 4/2, 3/2, 4/4	51.6	1 (6) 1.97 (85)
45)	DC	3 3/2, 4/2, 5/2	75	—
46)	DC	3 4/2, 3/2, 5/2	89.5	1 (100)
47)	DC	3 3/2, 4/2, 5/2	59	1 (2) 1.58 (49) 3.11 (21)
48)	DC	3 4/2, 5/2, 3/2	86.5	1 (88)
49)	DC	4 3/2, 4/2, 5/2, 5/3	72.1	1 (6) 1.62 (54) 2.97 (25)
50)	DC	3 5/2, 4/2, 3/2	72.5	1 (22) 1.81 (54)
51)	DLC	3 4/2, 5/2, 5/3	71.5	1 (17) 2.15 (48)
52)	DC	4 4/3, 5/3, 5/4, 4/4	72	1 (85) 1.89 (24)
53)	DCIS	7 4/3, 4/2, 5/3, 5/2, 4/4, 5/4, 6/3	72.6	1 (84)
54)	DC	3 5/3, 6/3, 4/3	78.5	1 (23) 1.81 (44)
55)	DC	3 5/2, 6/2, 4/2	94.5	1 (55) 1.86 (30) 3.47 (5)
56)	DC	4 4/2, 5/2, 3/2, 6/2	84.5	1 (5) 1.82 (54)
57)	DC	4 5/2, 6/2, 4/2, 5/3, 6/3	93.5	1 (94)
58)	DC	3 6/3, 6/4, 6/2	87.7	1 (62) 1.44 (28)
59)	DC	6 6/2, 5/2, 6/3, 5/2, 5/3, 7/3	83	1 (19) 1.90 (70)
60)	DC	>10 n.d.	86	1 (13) 2.54 (75)
61)	DC	>10 n.d.	97.5	1 (79)

FISH results and DNA index in three breast cancer samples with abnormalities and without under-representation of the 1p36 region

62)	DLC	1 2/3	8	—
63)	DC	1 3/3	13	1 (40) 1.99 (53)
64)	DC	3 3/3, 4/4	14.8	1 (54) 1.95 (22)

than in group 1 samples (t test, $p < 0.001$). Both probes were over-represented in nine samples. Excluding cases 60 and 61, which had a very high number of spots and various cell populations, we detected two to seven different cell populations in each sample.

Very large signals or blurred spots were frequently observed on hybridisation with pUC 1.77, making interpretation difficult, with the result that fewer cells were informative.

Three tumours had an abnormal number of signals per nucleus, although the p1-79 probe was not under-represented (cases 62–64, table 3). In case 62, a cell population with a greater number of p1-79 than pUC 1.77 signals was found in 12% of nuclei. This was the only abnormality detected. In two samples (cases 63 and 64) both probes were over-represented to the same extent. No cell population with relative 1p36 under-representation was found.

DNA PLOIDY

Fifty five specimens were investigated by cytofluorimetry; 24 (42%) were diploid and 31 (58%) were aneuploid. In three samples, two aneuploid stem-lines were detected. The DI value in the aneuploid tumours ranged from 1.34 to 3.47; 15 were tetraploid ($DI = 1.8$ – 2.20).

RELATION BETWEEN CHROMOSOME 1 ABERRATIONS AND DNA PLOIDY

Abnormalities were detected in 17 of 24 diploid samples. Ten were from group 1 and seven from group 2.

Only one aneuploid sample (case 6, table 1) did not show any chromosome 1 abnormalities by FISH. Five aneuploid samples were detected in group 1 and 23 in group 2 (χ^2 test, $p < 0.001$). Two cases with abnormalities but not under-representation of the 1p36 region were also aneuploid (table 3).

Discussion

Our results confirm that chromosome 1 aberrations occur frequently in breast cancer. Using a dual target FISH technique, we were able to detect chromosome 1 abnormalities in 56 of 64 tumours.

Deletion of 1p36 was clearly demonstrated in 11 samples, in which a single p1-79 spot was detected in a large number of nuclei. Seven of nine samples examined by cytofluorimetry were diploid. These results suggest that deletion of the 1p36 region may be an early event in the pathogenesis of breast cancer.²⁶

By means of the dual target technique, any chromosome 1 abnormality which gives rise to an imbalance with under-representation of the 1p36 region can be detected. However, loss of the 1p36 region cannot always be demonstrated. In fact, in the present study only the relative imbalance between the 1q12 and 1p36 regions resulting from chromosome 1 aberrations could be revealed in many cases by this technique.

The presence of chromosome 1 aberrations in breast cancer has already been demonstrated by single target FISH using the pUC 1.77 probe.^{11 27 28} Our results show that the dual target technique greatly improves the detection of these alterations; tumour samples which have a normal number of pUC 1.77 signals and structural aberrations such as deletion of the 1p36 region can also be detected.

Chromosome 1 aberrations seemed to be more complex and occur more frequently in aneuploid tumours; a very high number of signals was observed in 30 of 31 aneuploid compared with 17 of 24 diploid tumours.

Aneuploid tumours have a very complex karyotype and are thought to represent the more advanced stages of breast cancer.²⁹ In our study most aneuploid samples showed numerous pUC 1.77 spots, high INF and many abnormal cell populations. These results are, therefore, consistent with previously reported data showing that the number of 1q12 regions correlates to the DI value in breast cancer²⁸ and that clonal heterogeneity is a frequent finding in aneuploid tumours.³⁰

Interestingly, we detected chromosome 1 aberrations in 17 of 24 diploid samples. These data confirm the findings of conventional cytogenetic studies in which chromosome 1 aberrations were detected in near diploid tumours^{31 32} and may help to explain the contradictory conclusions reported in the literature on the prognostic value of the DNA index in breast cancer.³³ Diploid tumours have in fact been found to have both a good^{34 35} and a bad prognosis.^{36 37}

It is not possible to detect all chromosomal aberrations present on a particular chromosome using cytofluorimetry. As chromosome 1 is the most frequently altered chromosome in breast cancer, the use of FISH, in conjunction with cytofluorimetry, may improve characterisation of DNA ploidy in breast cancer.

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