Loss of heterozygosity at chromosome 9p in ductal carcinoma in situ and invasive carcinoma of the breast

KL Marsh and JM Varley
CRC Department of Cancer Genetics, Paterson Institute for Cancer Research, Christie Hospital, Wilmslow Road, Manchester M20 9BX, UK

Summary Twenty-three cases of ductal carcinoma in situ (DCIS), ten of which had an associated invasive component, were studied for loss of heterozygosity (LOH) of microsatellite markers on chromosome 9p and the results compared with a panel of 20 invasive breast carcinomas. In addition to the gene encoding p16, chromosome 9p is also thought to contain other putative tumour-suppressor genes. If the three panels of breast tumours showed LOH of markers in this region this would suggest that such putative genes were important in breast carcinogenesis. By studying both preinvasive and invasive breast tumours, it should also be possible to gain further information about the relationship between lesions of different stages and to determine whether DCIS is indeed a precursor of invasive ductal carcinoma. Levels of LOH were low in the invasive-only set of tumours. Surprisingly, considerably higher levels of loss were observed in the tumours with an in situ component. Also, much heterogeneity was observed between different DCIS ducts or invasive tumour and DCIS from the same case.

Keywords: ductal carcinoma in situ; microdissection; loss of heterozygosity; heterogeneity

Breast cancer remains one of the most common cancers in the Western world and overall it is estimated that approximately 1 in 10 women will develop this disease at some point in her life (Stratton and Wooster, 1996). Therefore, identification of environmental, biochemical and genetic factors that may be important in the aetiology and progression of this disease is essential in order to improve prevention, diagnosis and therapy. Conventionally, invasive cancer is regarded as developing through a series of events morphologically recognized as hyperplasia, atypical hyperplasia and in situ carcinoma, although for breast cancer this pathway of intermediate stages may be discontinuous and is certainly not universal. Although a variety of precursors of malignancy may be present in tissue surrounding invasive carcinoma, it is not uncommon for a cancer to be found in isolation, with no evidence of a non-infiltrating component in the area and no hint as to the nature of the progenitors of the disease. A close relationship between the preinvasive lesion ductal carcinoma in situ (DCIS) and invasive breast carcinoma has been postulated, as DCIS is frequently present in tissues adjacent to breast cancer (Ottesen et al., 1992; Schwartz et al., 1992). Furthermore, invasive breast cancers in women with DCIS generally occur in the same region of the same breast in which DCIS was originally identified (Lagios, 1993). By studying specific genetic alterations present in both preinvasive and invasive breast cancer, it was hoped to learn more about the relationship between these different stages.

In primary human breast tumours, loss of heterozygosity (LOH) represents the most frequent type of genetic alteration (Callahan et al., 1993). The assumption that LOH unmasks recessive alleles involved in tumorigenesis enables LOH analysis to be carried out on tumour DNA vs normal DNA, to determine the possible location of tumour-suppressor genes.

The chromosome region 9p21–p22 has attracted much research interest over recent years because of the identification, in 1994, of a new gene p16 (also known as MTS1, for multiple tumour-suppressor 1, INK4a and CDKN2, for inhibitor of cyclin-dependent kinase). The discovery that the gene is homozygously deleted in a large number of human tumour cell lines (Kamb et al., 1994; Nobori et al., 1994) suggested that p16 was behaving as a tumour-suppressor gene. The p16 protein had already been described as an inhibitor of cyclin-dependent kinases, which are themselves key regulators of the cell division cycle (Serrano et al., 1993). Loss of such an inhibitor would probably result in unregulated proliferation, thus reinforcing the support for p16 as a tumour suppressor. More recently, the availability of the p16–/− mouse (Serrano et al., 1996) and the observation that some familial melanoma families harbour germline point mutations or deletions within the p16 gene (Hussussian et al., 1994; Gruis et al., 1995) have provided further evidence that p16 is a tumour-suppressor gene. A second gene in this region, p15, also behaves as a tumour suppressor (Sonoda et al., 1995).

Regions of chromosome 9p have been found to be frequently rearranged or deleted in a range of primary tumours and often show LOH of genetic markers. There is evidence for a bladder cancer suppressor locus on chromosome 9p21 (Ruppert et al., 1993; Devlin et al., 1994; Orlow et al., 1994) with highest levels of loss at the IFNA gene cluster. Similar studies on other human tumours show significant LOH of the same region encompassing IFNA, including lung cancer (Mead et al., 1994; Merlo et al., 1994), glioma (Ichimura et al., 1994), renal cell carcinoma (Cairns et al., 1995) and oesophageal cancer (Tarmin et al., 1994). Some of these studies also describe high levels of loss both proximal and distal to IFNA, which are probably not targeting p16.

This study describes the assessment of LOH of microsatellite markers spanning chromosome 9p, to determine the location of putative tumour-suppressor genes involved in breast cancer.
MATERIALS AND METHODS

Tumour samples
Formalin-fixed, paraffin-embedded tumour tissue from 23 cases containing DCIS was obtained from Christie Hospital, Manchester, UK. Details of the histopathology and grade of these cases are given in Table 1. High molecular weight DNA had previously been prepared from frozen tumour material from a further 20 invasive breast cancer cases, for which there was also available the corresponding constitutional DNA, prepared from blood.

Microdissection and DNA extraction
Areas of normal tissue, ducts containing DCIS and, where present, invasive carcinoma were microdissected from a single dewaxed 20-μm paraffin section, with reference to an adjacent haematoxylin- and eosin-stained section. Care was taken not to contaminate tumour cells with normal cells. DNA was extracted by standard methods (Sambrook et al, 1989). When this supply of DNA had been used, the same ducts and regions of tissue that had originally been taken were subsequently microdissected from the equivalent area on serial sections, to enable experiments to be repeated.

PCR amplification
Primary PCRs were carried out in a volume of 20 μl, containing 340 μM each dNTP (Promega), 6 ng μl⁻¹ each of forward and reverse primer, 0.5–2 units Thermoprime plus DNA polymerase (Advanced Biotechnologies) and either 2–5 μl of DNA extracted from the microdissected tissue or 1 μl of blood–tumour DNA from the invasive breast cancer cases. Reactions were subjected to cycling conditions specific for each primer pair, with 25–50 cycles of denaturation at 94°C (typically 37 cycles for invasive tumour DNA and 43 cycles for DNA from microdissected material), annealing at temperatures of 55–62°C and extension at 72°C, each for 1 min. All polymerase chain reaction (PCR) programmes incorporated a denaturation step of 94°C for 4 min before cycling, with a final extension step of 72°C for 10 min after cycling.

An aliquot of 1 μl of product from the primary PCR was seeded into a 20-μl secondary PCR containing only one of the original primers, which had been end-labelled with [γ²³P]ATP using T4 polynucleotide kinase (200 ng of primer DNA and 1 μCi of ATP per reaction). The second round PCR programme consisted of just three cycles of 1 min at 94°C, 1 min at 55–62°C and 1 min at 72°C, followed by 2 min at 94°C, 5 min at 55–62°C and finally 10 min at 72°C.

Oligonucleotide primers
Oligonucleotide primers described in Kwiatkowski and Diaz (1992), Weissenbach et al (1992), Wilkie et al (1992) and Gyapay et al (1994), were used to amplify microsatellite markers of the (CA)₉ repeat type. Relative positions of these markers are shown in Figure 1.

| Case number | Histological classification | Nuclear grade | Grade of DCIS/inv |
|-------------|-----------------------------|---------------|-------------------|
| DCIS        |                             |               |                   |
| 2652        | Comedo, solid               | Intermediate  |                   |
| 2969        | Comedo, cancerization of lobules | Intermediate  |                   |
| 4419        | Comedo, cancerization of lobules, microinvasion | High |                   |
| 6050        | Solid, microcarcinoma. Duct ectasia, tiny focus of invasion | High |                   |
| 2238        | Comedo, cribriform. Lobular cancerization. Small focus of invasion | High |                   |
| 2239        | Comedo, solid. Lobular cancerization | High |                   |
| 2242        | Comedo, cribriform. Florid fibrocystic disease | High |                   |
| 3812        | Comedo                      | Intermediate/low |                   |
| 601         | Comedo. Occasional microinvasion | High |                   |
| 6092        | Comedo. Small focus of invasion | High |                   |
| 1960        | Solid. Fibrocystic change. Early microinvasion | High |                   |
| 2659        | Comedo. Some stromal invasion | High |                   |
| 4842        | Solid. Cancerization of lobules | Low |                   |
| DCIS + invasive |                           |               |                   |
| 3144        | Micropapillary. Invasive carcinoma. Fibrocystic disease | High | Intermediate/grade II |
| 6457        | Comedo, micropapillary. Invasive | High | grade II |
| 452         | Micropapillary. Intersitial ductal carcinoma | Intermediate/grade II |                   |
| 3041        | Cribriform, solid. Tubular carcinoma | Intermediate/grade I |                   |
| 4410        | Comedo. Infiltrating ductal carcinoma | Intermediate/grade III |                   |
| 6256        | Cribriform. Infiltrating ductal carcinoma | Intermediate/grade II |                   |
| 6384        | Comedo. Infiltrating ductal carcinoma | High | grade II |
| 613         | Cribriform. Invasive carcinoma | Low |                   |
| 2753        | Cribriform, solid. Infiltrating ductal carcinoma | Low–intermediate/II |                   |
| 2951        | Micropapillary. Intersitial ductal carcinoma | High | grade III |

Invasive cancers graded according to WHO guidelines. DCIS characterized using criteria based on nuclear grade.
The results of the LOH study for each of the three separate tumour sets, invasive-only tumour, DCIS-only and invasive tumour with a DCIS component, are represented in Table 2. In addition, the percentage of informative ducts showing LOH was recorded for each marker (\% loss).

Invasive cases
As can be seen in Table 2a, eight of the twenty infiltrating carcinomas show loss of at least one marker on chromosome 9p. Of these cases, five show loss of more than one marker (129, 219, 330, 343 and 353), with regions of retention in between. This suggests the presence of interstitial deletions, rather than the complete loss of the short arm.

Only two tumours (330 and 343) show loss of one or both markers that flank \( p16 \) (\( IFNA \) and D9S171). Case 330 demonstrates allelic imbalance at a number of markers across chromosome 9p, including a reduction in intensity of the lower allele at D9S285 (Figure 2A) and D9S1846/AFM325zh9 (Figure 2B) and complete loss of the upper allele at markers \( IFNA \) and D9S171 (Figure 2C and D). The adjacent marker D9S1679/AFM337b5 shows a reduction in intensity of the upper allele and there is retention of the more proximal marker D9S104 (Figure 2E and F). The fact that there is clear loss of the markers flanking \( p16 \) indicates that the DNA template is relatively free from contaminating normal cells and that all cells may have undergone a targeted loss of \( p16 \). However, the presence of allelic imbalance rather than clear loss at other markers might suggest that only a proportion of cells carry a deletion of these particular markers. This could be explained if loss of the \( p16 \) region was a relatively early event in the tumour’s development, followed by the divergence of subclones, some of which undergo loss of other markers on chromosome 9p at a later stage.

Levels of loss at most other markers are also relatively low and may be explained by a background level of random genetic loss, which is generally considered to be approximately 15\%. However, markers D9S1679 and D9S165 are lost in approximately 20\% of informative cases and marker D9S285 is lost in 33\% of cases, which may indicate the presence of putative tumour-suppressor genes in these regions.

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Detection of LOH
Radioactive PCR products were run on a denaturing 6\% polyacrylamide gel at 75 W for 2–4 h. Dried gels were exposed to medical X-ray film that was developed by being placed in Kodak D19 developer and Ilford Hypam fixer followed by thorough washing with water. For each case the autoradiogram was studied and DNA from both normal and tumour material was assessed for differences in the intensity of the alleles between the two. Complete loss or reduction in intensity of over 40\% (Hoggard et al, 1995) of one allele in the tumour relative to normal was considered to be LOH. For those cases that appeared to show loss of a particular marker, primary and secondary PCRs were repeated to confirm the result. To ensure that results were not caused by PCR artefacts, this was done using both the original DNA extract (when available) and DNA prepared from material freshly microdissected from the same duct on an adjacent serial section. A representative number of cases showing retention were also repeated, to confirm reproducibility of this technique.

RESULTS
The results of the LOH study for each of the three separate tumour sets, invasive-only tumour, DCIS-only and invasive tumour with a DCIS component, are represented in Table 2. In addition, the percentage of informative ducts showing LOH was recorded for each marker (\% loss).
### Table 2  Results of loss of heterozygosity (LOH) studies on chromosome 9p in (a) invasive breast cancers, (b) DCIS and (c) cases with both DCIS and invasive cancer

#### (a) Invasive breast cancer cases

| Case     | Locus | 76 | 127 | 129 | 139 | 161 | 163 | 166 | 219 | 228 | 330 | 343 | 344 | 353 | 364 | 369 | 370 | 374 | 389 | 395 | 413 | % Loss |
|----------|-------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| D9S285   |       |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 33.3 |
| D9S156   |       |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 17.6 |
| AFM121yf1| (D9S1782) |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 0    |
| D9S162   |       |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 11.1 |
| AFM352zh9| (D9S1846) |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 12.5 |
| INFA     |       |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 11.8 |
| D9S171   |       |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 7.7  |
| AFM377b5 | (D9S1679) |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 20   |
| AFM050y1 | (D9S1875) |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 0    |
| D9S104   |       |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 18.75|
| D9S165   |       |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 7.1  |
| AFM155xh4| (D9S1788) |   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 7.1  |

#### (b) DCIS cases

| Case     | Locus | 2652 | 2969 | 4419 | 6050 | 2238 | 2239 | 2242 | 3812 | 601 | 6092 | 1960 | 2659 | 4842 | % Loss |
|----------|-------|------|------|------|------|------|------|------|------|-----|------|------|------|------|------|
| D9S285   |       |     |      |      |      |      |      |      |      |     |      |      |      |      |      | 45.5  |
| D9S156   |       |     |      |      |      |      |      |      |      |     |      |      |      |      |      | 28.6  |
| AFM121yf1| (D9S1782) |   |      |      |      |      |      |      |      |     |      |      |      |      |      | 0     |
| D9S162   |       |     |      |      |      |      |      |      |      |     |      |      |      |      |      | 40    |
| AFM352zh9| (D9S1846) |   |      |      |      |      |      |      |      |     |      |      |      |      |      | 50    |
| INFA     |       |     |      |      |      |      |      |      |      |     |      |      |      |      |      | 50    |
| D9S171   |       |     |      |      |      |      |      |      |      |     |      |      |      |      |      | 33.3  |
| AFM377b5 | (D9S1679) |   |      |      |      |      |      |      |      |     |      |      |      |      |      | 50    |
| AFM050y1 | (D9S1875) |   |      |      |      |      |      |      |      |     |      |      |      |      |      | 40    |
| D9S104   |       |     |      |      |      |      |      |      |      |     |      |      |      |      |      | 35.7  |
| D9S165   |       |     |      |      |      |      |      |      |      |     |      |      |      |      |      | 42.9  |
| AFM155xh4| (D9S1788) |   |      |      |      |      |      |      |      |     |      |      |      |      |      | 38.9  |
Table 2 (Cont.)

| Case | Locus | DCIS/invasive cases | % Loss |
|------|-------|---------------------|--------|
|      |       | 3144                |        |
|      |       | 6457                |        |
|      |       | 4842                |        |
|      |       | 3041                |        |
|      |       | 4410                |        |
|      |       | 6294                |        |
|      |       | 6296                |        |

- **LOH at chromosome 9p in DCIS and invasive breast cancer**

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Six of the thirteen DCIS cases studied demonstrated LOH of at least one marker flanking p16 (IFNA or D9S171) that could potentially have arisen as a result of targeted loss of p16 accompanied by loss of additional markers. The IFNA marker was lost in 50% of ducts examined. High levels of loss comparable with this were also found at markers both proximal and distal to p16. As had been observed in the invasive cases, markers D9S165 and D9S285 were among those affected with the highest frequency, being lost in >40% of cases. However, D9S1846 and D9S1679, like IFNA, were lost in 50% of cases, although the number of ducts assessed was only relatively small in each case. Again, these high levels of loss may indicate the location of putative tumour-suppressor genes.

**DCIS cases with an invasive component**

A number of markers spanning chromosome 9p were successfully amplified from both comedo DCIS DNA (C) and infiltrating ductal carcinoma DNA (I) from case 4410. As the results in Table 2c and Figure 4 show, concordance between these two components was found in every case. These results suggest that, in this case at least, the DCIS and the invasive tumours are intimately related, the most likely explanation being that the DCIS was the precursor lesion. However, a more complex pattern of genetic alterations is observed in other cases, with the preinvasive and invasive components not showing the same changes. For example case 6256 demonstrates retention of both alleles of the IFNA marker in the cribriform DCIS but loss of the upper allele in the invasive component, perhaps indicating that loss of this particular region is important in the progression from in situ to invasive carcinoma (Figure 5). A slightly more complex situation is shown in Figure 6. As had been observed in the subgroup of tumours containing DCIS only, when numerous ducts were microdissected from cases containing both DCIS and carcinoma, retention of both alleles was found in some ducts, with LOH in others. Also, as noted previously, losses in different ducts may affect different alleles, reinforcing the idea that DCIS shows considerable genetic heterogeneity.
Despite the uncertainty regarding the relevance of losses flanking p16, the levels of loss at other markers on chromosome 9p, in the DCIS and DCIS/invasive cases in particular, suggested the possible location of other tumour-suppressor genes both proximal and distal to p16. D9S285 was lost at consistently high frequency (>30%) across the three tumour sets, as were markers D9S1679 and D9S165. Also, although levels of loss in the invasive panel of tumours were low, the markers D9S1788 and D9S162 were lost in approximately 40% or more of cases in the remaining two groups of tumours. Other studies looking at LOH of microsatellite markers spanning chromosome 9p in various tumours have found similar results, with many losses centred around the IFNA marker, but also extending proximally and distally (Olopade et al, 1993; Coleman et al, 1994; Neville et al, 1995).

One purpose of the study had been to attempt to gain more information about the natural history of breast cancer and the molecular changes accompanying progression. This study and others (James et al, 1997) have confirmed that genetic changes found in DCIS lesions are similar to those found in invasive breast carcinoma. Furthermore, a recent paper has also described some changes in morphologically normal tissue adjacent to breast carcinomas (Deng et al, 1996).

One significant conclusion to be drawn from this work is that, even at the preinvasive stage of DCIS, breast cancer is a heterogeneous disease in which the pathway of changes accompanying progression is far from clear. Although the results indicated that some DCIS lesions are likely to be the precursors of invasive cancer, because both components showed identical allele loss for particular markers, this is not true for all DCIS, some of which have quite distinct genetic alterations to those of the invasive component. Reports in the literature have described both situations. A study by Zhuang et al (1995) assessed allelic imbalance of markers on chromosome 11q13 in microdissected in situ and invasive human breast cancer. This provided evidence for a tumour-suppressor gene in this region that may be important in breast cancer development, and also indicated that invasive breast cancer arises from in situ lesions. However, similar studies looking at allelic imbalance have described intratumour heterogeneity with the probable existence of subpopulations of tumour cells (Chen et al, 1992; Bonsing et al, 1993). An extension of this has been noted in studies similar to this one, in which several ducts from the same case were treated independently. In agreement with the results described in this study, heterogeneity with respect to allele loss was found in different neoplastic foci of in situ cancer and in some cases allelic losses present in in situ regions were not conserved in the progression to invasive tumour (Munn et al, 1995; 1996; Fujii et al, 1996).

It remains surprising that the degree of loss observed in the invasive-only set of tumours was so much less than that found in the other two sets. It might be expected that because the DCIS lesions show such a high level of loss of markers on chromosome 9 that they may show a correspondingly high frequency of loss of other markers across the genome. However, this has been shown not to be the case (Aldaz et al, 1995; Radford et al, 1995), with allelotype studies on DCIS showing levels of loss less than those found in invasive breast carcinomas. Although direct comparisons between the three sets of tumours cannot be made and numbers are small, the results of this study may suggest that this particular set of invasive lesions is fundamentally different to those lesions that have an intraductal component. A similar result has previously
Figure 6  Both loss and retention of the same marker in different ducts from the same case. (A) The comedocell duct (C) from case 6457 shows retention of the upper allele of marker D9S162 in contrast to the micropapillary duct (M), which shows clear loss of this allele. Similarly, in case 2753 clear LOH is seen in the cribriform duct (Cr), in contrast to the retention of both alleles seen in the solid (S) duct. (B) This phenomenon is not confined to ducts of different subtype, as different comedo ducts from case 6457 show different results when the genotype of the IFNA marker is assessed. (C) A further level of heterogeneity exists between individual ducts from the same case. Case 6457 demonstrates retention of both alleles at marker D9S104 in duct C2. However, ducts C1 and C2 show loss of opposite alleles, indicating that they have undergone distinct genetic alterations affecting different chromosomes.

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