Chromosome 3p allele loss in early invasive breast cancer: detailed mapping and association with clinicopathological features

A Martinez, R A Walker, J A Shaw, S J Dearing, E R Maher, F Latif

Abstract

Aims—Chromosome 3p allele loss is a frequent event in many common sporadic cancers including lung, breast, kidney, ovarian, and head and neck cancer. To analyse the extent and frequency of 3p allelic losses in T1N0 and T1N1 invasive sporadic breast cancer, 19 microsatellite markers spread along 3p were analysed in 40 such breast carcinomas with known clinicopathological parameters.

Methods—Loss of heterozygosity analysis was carried out using 3p microsatellite markers that were non-randomly distributed and chosen to represent regions that show hemizygous and/or homozygous losses in lung cancer (lung cancer tumour suppressor gene region 1 (LCTSGR1) at 3p21.3 and LCTSGR2 at 3p12), and regions demonstrating suppression of tumorigenicity in breast, kidney, lung, and ovarian cancer.

Results—Allelic loss was seen at one or more loci in 22 of these clinically early stage sporadic breast tumours, but none had complete 3p allele loss. Several regions with non-overlapping deletions were defined, namely: (1) 18 tumours showed loss at 3p21–22, a physical distance of 12 Mb; (2) 11 tumours showed loss at 3p12 within a physical distance of 1 Mb, this region is contained within LCTSGR2; (3) six tumours showed loss at 3p25–24, including the von Hippel-Lindau (VHL) locus; (4) five tumours showed loss at 3p14.2, including the fragile histidine triad (FHIT) locus.

Conclusions—This is the largest study to date defining the extent and range of 3p allelic losses in early stage invasive breast cancer and the results indicate that region 3p21–22 containing LCTSGR1 and a region at 3p12 within LCTSGR2 are the most frequent sites of 3p allelic loss in these breast carcinomas. This suggests that tumour suppressor genes located in these regions may play important roles in the development of breast cancer. There was an association between increasing 3p allelic loss and increasing tumour grade and loss of progesterone (p = 0.0098) and oestrogen (p = 0.0472) receptor expression, indicating a link between 3p allelic loss and the regulation of differentiation.

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Section of Medical and Molecular Genetics, Department of Paediatrics and Child Health, University of Birmingham, The Medical School, Edgbaston, Birmingham B15 2TT, UK
A Martinez
E R Maher
F Latif

Breast Cancer Research Unit, University Of Leicester, Clinical Sciences, Glenfield Hospital, Groby Road, Leicester LE3 9QP, UK
R A Walker
J A Shaw
S J Dearing

Correspondence to:
Dr Latif
flatif@hgmp.mrc.ac.uk

In the Western world breast cancer is the most prevalent malignancy in women. The incidence of breast cancer is rising and it is estimated that one in 10 women will develop breast cancer during her life time.[1] An understanding of the genetic alterations involved in breast cancer development and progression may aid earlier detection and management.

Certain alterations, such as amplification of the oncogene ERBB2, can be found in a proportion of both in situ and invasive breast cancers, as can mutations of the tumour suppressor gene (TSG) p53 at 17q13, suggesting that they could play a role in the development of these tumours. Alterations to other oncogenes and TSGs, such as MYC and RB1, are associated with more advanced disease.[2] Loss of heterozygosity (LOH) on chromosomes 1, 3p, 6q, 7q, 8p, 11p, 13q, 17p, 17q, 18q, and 22q has been reported in breast carcinomas and other tumours (reviewed in Bieche and Lidereau[3] and Schwab[4]), indicating a role for TSGs located in these regions in the development and progression of different cancers. For familial breast cancer, two major genes have been isolated, BRCA1 at 17q21 and BRCA2 at 13q12–13 (reviewed in Buchholz and colleagues[5] and Yang and Lippman[6]), and a third locus (at least) is also thought to exist.[7 8] BRCA1 and BRCA2 do not show inactivating mutations in sporadic breast tumours, and their role in sporadic cancers is not known.

By means of hemizygosity and homozygosity mapping, cytogenetic analysis, and functional studies, distinct regions on 3p (3p25–26, 3p 21–22, 3p14.2, and 3p12) have been shown to be important for the development of several common sporadic cancers including lung, breast, kidney, ovarian, cervical, and head and neck cancer (reviewed in Kok and colleagues[9]).[10–13] The region 3p25 contains the von Hippel-Lindau (VHL) TSG,[14 15] which is inactivated in patients with von Hippel-Lindau disease and approximately 70% of sporadic clear cell renal carcinomas.[16–19] However, mutations of VHL are rare in other common sporadic cancers that show 3p allele loss, such as lung and gonadal tumours.[17 20] The fragile histidine triad (FHIT) gene at 3p14.2 undergoes homozygous deletions and alterations in its mRNA in many sporadic cancers (reviewed in Huebner and colleagues[21] and Sozzi and colleagues[22]). High amounts of allele loss at the FHIT locus have been found in low grade ductal carcinoma in situ of the breast and the well differentiated tubular carcinomas,[23] suggesting that alterations at the FHIT locus may be

important in development of low grade breast cancer.

We have previously reported homozygous 3p deletions in sporadic breast cancers. The region at 3p21.3 (lung cancer tumour suppressor gene region 1; LCTSGR1) is defined by four overlapping homozygous deletions in three small cell lung cancer cell lines and one primary breast tumour.[24–27] The 3p12 region (LCTSGR2) contains two overlapping homozygous deletions in small cell lung cancer cell lines and one breast tumour cell line.[28] Several genes have been isolated from LCTSGR1 and one from LCTSGR2, but so far none shows frequent inactivating mutations in lung cancer.[27 28] Recently, a small study (n = 8) demonstrated 3p allelic loss in benign breast lesions preceding invasive breast cancer.[29] Another study demonstrated 3p loss in normal tissue adjacent to breast carcinomas, the most frequent loss being 3p22–25.[30] However, only a low frequency of 3p loss has been found in comparative genomic hybridisation studies of ductal carcinoma in situ.[31]

We have analysed 3p allelic losses in T1N0 and T1N1 sporadic invasive breast carcinomas to determine their extent and frequency. We focused on specific 3p regions, (3p25–26, 3p21–22, 3p14.2, and 3p12) implicated in tumorigenesis in breast and other cancers. We found 3p loss in most of these breast tumours, and the two most frequently lost regions on 3p included LCTSGR1 and LCTSGR2. In addition, a trend was found between 3p allelic loss, higher tumour grade, and loss of oestrogen and progesterone receptors (ER and PR, respectively), suggesting that there are genes on 3p that are associated with differentiation and that their loss results in breast cancers with more aggressive features.

Materials and methods

PATIENTS AND SAMPLES

A total of 40 invasive breast carcinomas (39 infiltrating ductal carcinomas and one infiltrating lobular carcinoma) were studied. Twenty five were detected by mammographic screening and the others had presented symptomatically. Carcinomas were excised at Glenfield Hospital NHS Trust between July 1995 and July 1997. Tumours of 20 mm or less in maximum diameter were examined (range, 10–20 mm; mean, 17), and 15 had nodal metastases (T1N0 or T1N1, no more than three lymph nodes involved in positive cases). None of the tumours was from a woman with a known family history of breast or other cancers.

All tissues were fixed in 4% formaldehyde in saline for 18–36 hours. After slicing, selected blocks were processed through graded alcohols and xylene to paraffin wax.

The carcinomas were reported according to the Royal College of Pathologists' working party guidelines (1990). Infiltrating ductal carcinomas were graded using the modified Bloom and Richardson system.[32] All tissue histological assessments were performed by RAW.

ER and PR immunohistochemistry was undertaken as described previously.[33]
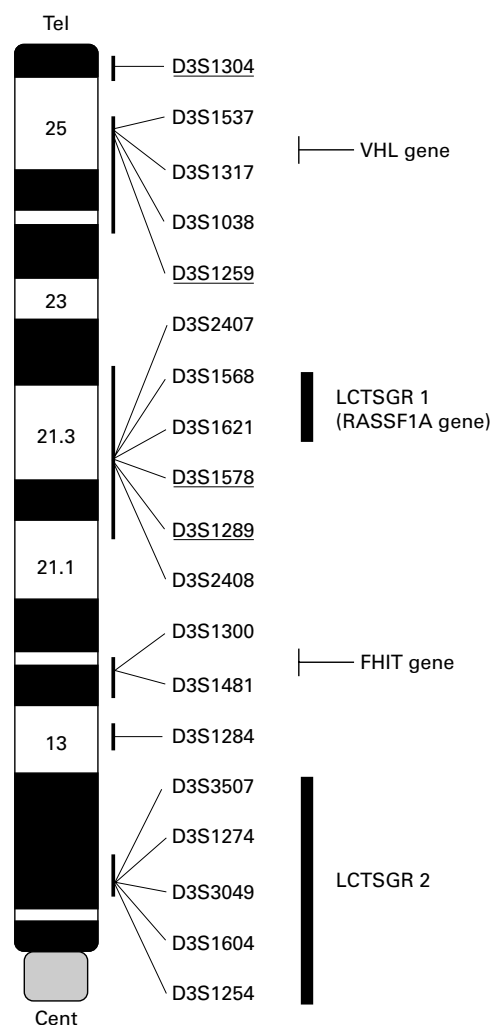

Figure 1   Summary of polymorphic markers and regions of interest on chromosome 3p. Genetic markers are listed in descending order from telomere to centromere according to published maps (UDB) and websites (http:// apollo.uthscsa.edu/) and approximate cytogenetic (ideogram) positions. The regions LCTSGR1 and LCTSGR2 implicated in lung cancer development at 3p21.3 and 3p12 are also shown. The underlined markers represent loci shown to be important for suppression of tumorigenicity in an ovarian tumour cell line.[11 40] The positions of the VHL (von Hippel-Lindau), RASSF1A and FHIT (fragile histidine triad) genes are also shown.

DNA EXTRACTION AND MICRODISSECTION FROM PARAFFIN WAX EMBEDDED SECTIONS

Formalin fixed, paraffin wax embedded tissue from breast tumours and non-involved nodes served as the source of tumour and normal DNA, respectively. For each tumour–normal pair, DNA was extracted from 10 µm thick paraffin wax embedded sections, as described previously.[34]

MICROSATELLITE REPEAT ANALYSIS

Polymerase chain reaction (PCR) amplification of dinucleotide, trinucleotide, and tetranucleotide microsatellite sequences was carried out. Nineteen markers were selected spanning the regions of interest on 3p. All are available through Genome Database with the exception of new primers for the D3S1621 locus (forward primer, 5'-CCTCACTACTCCTGG AATTG-3'; reverse primer, 5'-CCAAGGAA

GGGTTTTA CTTA-3'; PCR product size 140 bp, annealing temperature 55°C). LOH analysis was carried out as described previously.[11] Electrophoresis was carried out for two to four hours at 90 W constant power to achieve adequate separation of alleles. After drying the gel was exposed to *x* ray film (Fuji, Tokyo, Japan).

We defined LOH as a complete absence of, or significantly decreased signal intensity of, one of the constitutional alleles in tumour DNA as determined by visual examination.

STATISTICAL ANALYSIS

Comparisons were made by Fisher's exact test and the χ^2 test as appropriate. p Values of < 0.05 were taken as significant.

Results

LOH ANALYSIS USING 3P MICROSATELLITE MARKERS

Figure 1 shows the location of the 19 microsatellite markers from 3p used to screen the 40 tumour–normal DNA pairs of breast carcinomas. The markers are non-randomly distributed and were chosen to represent regions showing hemizygous or homozygous losses and regions that show evidence of suppression of tumorigenicity in several common sporadic cancers including lung, breast, kidney, and ovary (fig 1).

Allelic loss of one or more markers at 3p was seen in 22 tumours. None showed loss of every informative marker and 18 tumours showed no loss of informative markers (fig 2). The 22 tumours showing partial losses of 3p markers were analysed to identify regions of minimal overlapping deletions (fig 2). The highest loss

(18 of 38 informative tumours) was seen at 3p21–22, between D3S2407 and D3S2408. This interval includes the region LCTSGR1, represented by microsatellite markers D3S1568 (equivalent to D3S4615) and D3S1621 (equivalent to D3S4623) and the region shown to be functionally important in ovarian cancer development. Within the region 3p21–22, the highest loss was observed for D3S2408 (13 of 25 informative tumours). The physical distance of this region bounded by D3S2407 and D3S2408 is 12 Mb (according to the unified database; UDB). Within this large region, smaller regions of overlapping allelic loss identified a region between D3S1289 and D3S2408, with a physical distance of 0.65 Mb, as the candidate region (tumours 20 and 14 both show loss at 1289 and retention at 2408, whereas tumours 9, 11, and 19 show retention at 1289 and loss at 2408) (figs 2 and 3). Four tumours showed loss only at 3p21–22 and retention of all informative markers at other 3p regions (tumours 17, 11, 19, and 20; fig 2). The next most frequently lost region was at 3p12, within the LCTSGR2 region, which was lost in 11 of 39 informative tumours. The highest 3p12 loss was seen for D3S1604 (five of 22 informative tumours); the markers D3S3507, D3S1274, D3S3049, and D3S1604 are located within < 1 Mb of each other. Nine tumours showing LOH at 3p12 also showed loss of distal markers, the remaining two tumours showing loss at 3p12 only were not informative for all distal markers. The region at 3p25–24, which includes the VHL locus, was lost in six tumours; however, all of these tumours also showed LOH at 3p21 and/ or 3p12/3p14. Five tumours showed LOH at
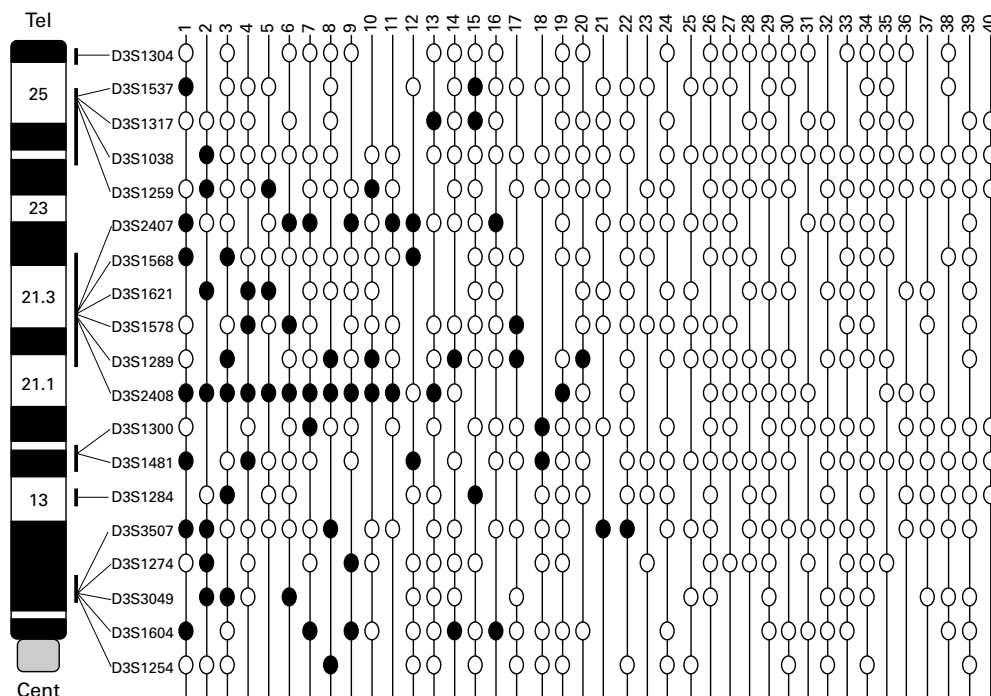

Figure 2   Summary of loss of heterozygosity (LOH) analysis. LOH pattern for all 40 early sporadic breast tumours (39 infiltrating ductal carcinomas and one infiltrating lobular carcinoma) for 3p markers. Each column represents a tumour and each row represents a 3p microsatellite marker listed in descending order from telomere (D3S1304) to centromere (D3S1254). The cases are arranged from left to right in decreasing order of chromosome 3p deletions. Status of each 3p locus is indicated: black circles, loss; white circles, retention; no symbols, uninformative loci.

the FHIT locus at 3p14.2, but again four of these were accompanied by more distal and or proximal losses, and the one remaining tumour was not informative for all distal and proximal markers.

ASSOCIATION WITH CLINICOPATHOLOGICAL PARAMETERS

A trend was seen between increasing 3p loss and higher tumour grade. Two of the eight grade 1 tumours, 11 of the 21 grade 2 tumours,
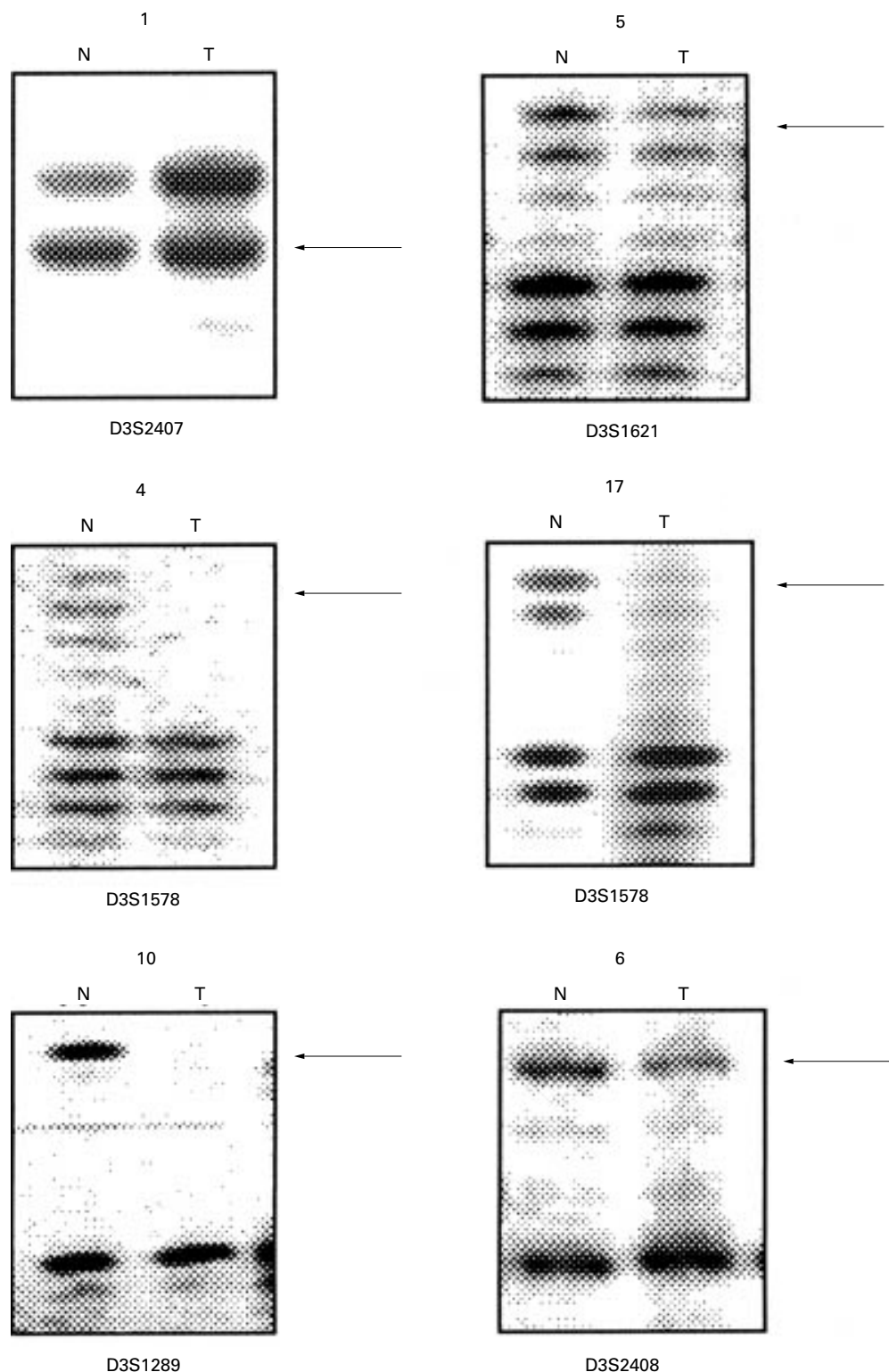

Figure 3 Representative examples of 3p loss of heterozygosity (LOH). Representative autoradiographs showing allelic losses for the markers at 3p21–22 (D3S2407, D3S1621, D3S1578, D3S1289, and D3S2408) in breast tumours. For each autoradiograph the case number is on the top. N, normal; T, tumour DNA. Arrowheads represent LOH.

Table 1   3p loss of heterozygosity (LOH), together with grade, lymph node status, progesterone receptor (PR) status, and oestrogen receptor (ER) status

	LOH (number)	Retention (number)
	Any locus on 3p	
Grade		
1	2	6
2	11	10
3	8	3
Lymph node status		
–	14	11
+	7	7
p = 0.7496 (NS)		
Hormone receptor PR		
–	9	1
+	10	15
p = 0.0098		
Hormone receptor ER		
–	7	1
+	12	15
p = 0.0472		

The results show an association between 3p LOH and increasing tumour grade, although this is not significant. There was no correlation between LOH and lymph node status. There was a significant correlation between loss of PR and ER expression (PR, p = 0.0098; ER, p = 0.0472) and loss at any 3p locus.

and eight of the 11 grade 3 tumours showed 3p loss. Although the above figures are not significant, they do demonstrate a trend between 3p loss and increasing tumour grade.

Next, we investigated whether there was an association between tumour PR and ER expression and 3p allele loss. When the whole 3p arm was analysed we found that PR negative status was significantly more frequent in carcinomas with LOH at any 3p marker (nine of 19) than in those without 3p LOH (one of 16) (p = 0.0098). A significant association was also found for ER negative status and 3p loss (seven of 19) compared with tumours without 3p loss (one of 16) (p = 0.0472) (table 1). Furthermore, we found that two of the three carcinomas (tumours 11, 17, and 19) that had only 3p21–22 loss and known receptor status had loss of PR and ER expression, whereas the remaining tumour was positive for both receptors. This suggests a correlation between 3p21–22 allele loss and lack of PR and ER expression. The presence or absence of cancer cells in the lymph nodes is an important prognostic parameter; patients with few or no positive nodes have a far better prognosis than those with many. In our series of early stage invasive breast tumours, 14 of 25 lymph node negative tumours had loss of 3p markers, as did seven of 14 lymph node positive tumours. Hence, there was no correlation between lymph node status and 3p loss (p = 0.7496) (table 1).

Discussion

We undertook high resolution deletion mapping on chromosome 3p in T1N0 and T1N1 invasive breast cancers to determine the frequency and importance of 3p loss and to map the precise regions on 3p that may contain TSGs important in breast cancer progression. We found the following: (1) a high incidence of 3p loss in this defined group of sporadic breast cancers; (2) evidence for two major candidate regions for 3p breast TSGs; (3) a trend between 3p allele loss and tumour grade; and (4) a significant correlation between 3p loss and loss of PR and ER expression. Although several other studies have analysed 3p LOH in breast cancer and reported LOH frequencies of 27–51%,[35–38] these studies included all tumour stages and did not use a high density of markers for candidate breast cancer TSG regions. We found that just over a half of early stage invasive breast tumours showed loss of one or more 3p markers and defined two minimal regions of loss that may contain TSGs involved in the progression of breast cancer. Within the group studied we saw a trend between 3p LOH and increasing tumour grade. A recent study using comparative genomic hybridisation analysis of unselected breast cancers reported higher 3p loss in grade 3 breast tumours than in grade 1 tumours.[39] These findings suggest that there are genes on 3p that are involved in the control of differentiation. Loss of these will result in the development of more aggressive cancers and this is confirmed by our finding of a significant correlation between chromosome 3p allele loss (most notably 3p21–22) and lack of PR and ER.

Multiple TSGs map to chromosome 3p and although we observed loss of one or more 3p markers in just over a half of the tumours, no tumour showed complete 3p LOH. We found that LOH was most frequent at 3p21–22. This region contains the LCTSGR1 (represented by microsatellite markers D3S1568 and D3S1621), which demonstrates overlapping homozygous deletions in lung and breast tumour cell lines.[24–27] Although the physical distance between the markers at borders of this region is 12 Mb, the smaller regions of overlapping allelic losses within this interval narrow the candidate region to 0.65 Mb. This region between D3S1289 and D3S2408 overlaps with one of three candidate regions for suppression of tumorigenicity of an ovarian tumour cell line (fig 1).[11 40] Multiple genes have been isolated from LCTSGR1,[27] but to date, inactivating mutations in these genes are absent or rare in human cancers. Very recently, we and others have identified a gene (RASSF1A) from LCTSGR1 at 3p21.3 that is epigenetically inactivated in most lung cancers and to a lesser extent in breast tumours.[41–43]

The second most frequent region of LOH in early breast cancer that we identified was at 3p12. Within this region, the marker D3S1604 gave the highest loss, and the candidate interval corresponded to the LCTSGR2 region, which is defined by the presence of homozygous deletions in two lung cancer cell lines.[28 44 45] LCTSGR2 also contains a homozygous deletion in one breast tumour cell line and this region is cloned in a 8 Mb yeast artificial chromosome contig. A candidate TSG, DUTT1, was isolated from this region. DUTT1 is a member of the NCAM family of genes, which includes the DCC (deleted in colorectal cancer) gene; however, DUTT1 mutations have so far not been identified in lung cancer and mutation analysis of DUTT1 in breast cancer has not been reported.[28 46] Recently, the 3p12 region implicated in renal cell carcinoma by microcell mediated tumour suppression

studies was shown to overlap with LCTSGR2.[47 48]

We also observed 3p LOH at 3p14.2 and 3p24–25 in our panel of breast tumours. However, loss in these regions was less frequent than at 3p21–22 and 3p12, and no tumour that was informative at 3p12 and 3p21 demonstrated LOH at 3p14.2 and not 3p12 or 3p21. Similarly, 3p24–25 LOH was always seen in tumours with coexisting 3p21–22 or 3p12 LOH. The VHL TSG maps to chromosome 3p25, but VHL gene mutations have not been identified in breast cancer. The 3p24–25 region is one of three candidate intervals for an ovarian cancer TSG; however, although a distal 3p TSG may be involved in some breast cancers, other 3p TSGs appear to be more important. We found five breast tumours that showed allelic losses at the FHIT locus. Abnormalities of FHIT mRNA transcripts and hemizygous and homozygous allelic losses at the FHIT locus have been reported in various cancers including lung, kidney, breast, and digestive tract cancers (reviewed in Huebner and colleagues[21] and Sozzi and colleagues[22]). Although the role of the FHIT gene in tumorigenesis is still controversial, our results suggest that other 3p TSGs are more important for breast tumorigenesis. The pattern of discontinuous regions of 3p LOH with frequent losses at 3p21–22 and 3p12 is similar to that reported in lung cancer and preneoplastic lung lesions.[12 49] The similarities between 3p LOH in lung and breast cancer suggest that the relevant 3p TSGs are involved in both tumour types.

In summary, we have demonstrated that regions on 3p (3p21–22 and 3p12) containing overlapping homozygous deletions in lung and breast tumours and tumour cell lines and a region involved in ovarian tumour suppression (3p21) show a high percentage of allelic losses in T1N0 and T1N1 invasive cancer, irrespective of node status, hence providing evidence for the involvement of genes residing at these regions in sporadic breast cancer development and progression. We also demonstrated increasing 3p loss with tumour grade and loss of ER and PR expression, resulting in the formation of more aggressive tumours. Larger studies are required to evaluate whether the 3p allelic losses described in this report can form a useful screening tool for identifying and understanding more aggressive disease.

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