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Detection of Allelic Imbalance Indicates That a Proportion of Mammary Hyperplasia of Usual Type Are Clonal, Neoplastic Proliferations

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SUMMARY: Previously, we developed methodology for studying allelic imbalance (AI) in preinvasive breast disease and showed that AI identified at various chromosomal loci in invasive carcinoma is already present in *in situ* carcinoma and atypical hyperplasia. We now extend this work by looking for AI in hyperplasia of usual type (HUT), apocrine cysts (AC), and papilloma (Pap) of the breast. HUT, AC, and Pap were identified in formalin-fixed, paraffin-embedded sections of benign breast biopsies and isolated using a microdissection technique. AI was investigated using polymorphic microsatellite markers and PCR.

AI was identified in 3/23 (13%) informative cases of HUT at 17q (D17S250), 2/43 (4.7%) at 17p (D17S796), 1/22 (4.5%) at 16q (D16S413), and 0/18 (0%) at 13q (D13S267). No particular histologic feature of HUT predicted the presence of AI. No examples of AC or Pap exhibited AI at any of the markers studied.

AI previously identified at various chromosomal loci in invasive carcinoma, *in situ* carcinoma, and atypical hyperplasia is present at low frequency in HUT in benign breast biopsies but not in AC or Pap. The possibility that AI in the latter could be masked by contamination from stromal and myoepithelial cells cannot, however, be excluded at this stage. At least a proportion of HUT thus appear to be clonal (neoplastic) rather than hyperplastic proliferations as their name suggests. The significance of AI in the pathogenesis of HUT or its subsequent progression to carcinoma is not yet clear and requires further investigation. *(Lab Invest 1996, 74:129–135)*

The delineation of genetic events involved in the transition from normal colonic epithelium to adenoma and colorectal carcinoma (Vogelstein *et al.*, 1988) has paved the way for investigation of the molecular events associated with the development of other solid neoplasms. The study of putative precursor lesions in the breast, however, is made more difficult by their microscopic size and ill-defined nature. Furthermore, benign changes within the human breast constitute a wide variety of lesions, the morphologic classification of which is still highly controversial.

Evidence that breast tumors can arise from precursor lesions in a multistep fashion is provided by the classic experiments on mice infected with the murine mammary tumor virus (DeOme *et al.*, 1959; Morris and Cardiff, 1987). Breast epithelial cells in these animals undergo nodular proliferation to form hyperplastic alveolar nodules, which when transplanted into cleared mammary fat pads, develop into tumors more frequently than normal breast tissue. Transformation is not, however, inevitable.

Evidence from human studies is indirect. Infiltrating carcinoma is often associated with *in situ* carcinoma and various forms of ductal and lobular hyperplasia, suggesting that they may be precursors of invasive carcinoma (IC) at different stages of oncogenesis. Further evidence comes from prospective studies, such as those conducted by Dupont and Page (1985), which showed that the relative risk of developing carcinoma in women with hyperplasia is increased particularly if it is atypical. The risk is approximately doubled in patients with a family history of breast cancer. These results support the proposal that hyperplastic lesions with and without atypia may be precursors of invasive cancer.

As a first step in trying to delineate the molecular events important in the evolution of breast cancer, we developed methodology to study allelic imbalance (AI) in microdissected fragments from paraffin-embedded material. We demonstrated that AI identified at various chromosomal loci in IC is already present not only in

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ductal carcinoma *in situ* (DCIS) (Stratton *et al.*, 1995) and lobular carcinoma *in situ* (LCIS) (Lakhani *et al.*, 1995) but also in atypical ductal hyperplasia (ADH) (Lakhani *et al.*, 1995), suggesting that this is also a clonal (neoplastic) proliferation. We now extend this work to investigate AI in benign proliferative breast lesions with two main aims: (i) to determine at which point along the putative pathway of evolution of human breast carcinoma AI first appears, and (ii) to determine if it is associated with any particular morphologic features that may help in classifying the various forms of proliferative change.

The PCR-based methodology employed for the study of loss of heterozygosity (LOH) does not allow differentiation between loss of one allele or gain of the other and hence, the preferred term, AI, is used throughout. However, in most of the cases described, the imbalance is so great that it is best interpreted as a loss with a small contaminating stromal cell population.

Results

The results presented include those cases in which at least one lesion and the normal tissue yielded DNA that was successfully amplified. Only a limited number of markers were examined because of constraints on the amount of tissue available for analysis and the need for repetition of results. The results are summarized in Table 1. Figs. 1, 2, and 3 illustrate the examples of hyperplasia of usual type (HUT), apocrine cysts (AC), and papilloma (Pap) together with their associated fluorescent traces.

Fifty-one cases of HUT were informative for one or more markers. AI was detected in 3/23 (13%) informative cases at 17q (D17S250), 2/43 (4.7%) at 17p (D17S796), 1/22 (4.5%) at 16q (D16S413), and 0/18 (0%) at 13q (D13S267). In 17 cases, more than one focus of hyperplasia was dissected from the same biopsy specimen. In four of these, AI was detected in at least one locus: (i) in the first, it was seen in one of three foci at 17p; (ii) in the second, it was present in one of five foci at 17q; (iii) in the third, it was detected at both 17q and 17p in one of four separate foci (Fig. 1); and (iv) in the last, two of three foci showed AI at 17q (Fig. 2).

Four cases of HUT had focally extensive areas with apocrine change within them. One of these showed evidence of AI but was difficult to interpret definitively, because the alleles were close together, and the imbalance in the ratios of the alleles fell short of what we usually accept as a positive result. The other three cases did not exhibit AI at any loci.

All cases of hyperplasia had detailed morphologic and cytologic assessment as described in the "Methods" section. The cases exhibiting AI did not, however, show any specific histologic features.

Thirty-four cases of AC were studied, 27 of which were informative at one or more markers. AI was not detected in any sample at any of the loci studied (Table 1, Fig. 2).

Eleven Pap were also studied, and eight were informative at one or more loci. None exhibited AI at any of the loci studied (Table 1, Fig. 3).

Discussion

Using a microdissection technique, polymorphic microsatellite markers, and PCR, we looked for AI in HUT, AC, and Pap of breast. We previously demonstrated AI in DCIS, LCIS, and ADH (Lakhani *et al.*, 1995; Lakhani *et al.*, 1995; Stratton *et al.*, 1995) at various chromosomal loci with a similar frequency to that identified in invasive ductal carcinoma, suggesting that AI at these loci represents relatively early events in breast carcinogenesis.

In the present study, we found AI in HUT with a frequency ranging from 0 to 13% at loci on chromosomes 13q, 16q, 17p, and 17q. One case of hyperplasia exhibited AI at loci on 17p and 17q, suggesting either two distinct regions of AI or possible loss of the entire chromosome. AI was not identified in any case of AC or Pap.

Table 1. AI in Benign Non-Atypical Breast Disease (Total Number of Cases Studied = 68)

	16q (D16S413)	17p (D17S796)	17q (D17S250)	13q (D13S267)
HUT (51 cases studied)				
No. exhibiting AI/No. Informative	1/22	2/43	3/23	0/18
% exhibiting AI	4.5%	4.7%	13%	0%
AC (34 cases studied)				
No. exhibiting AI/No. Informative	0/13	0/21	0/12	0/4
% exhibiting AI	0%	0%	0%	0%
Pap (11 cases studied)				
No. exhibiting AI/No. Informative	0/0	0/7	0/5	0/1
% exhibiting AI	—	0%	0%	0%

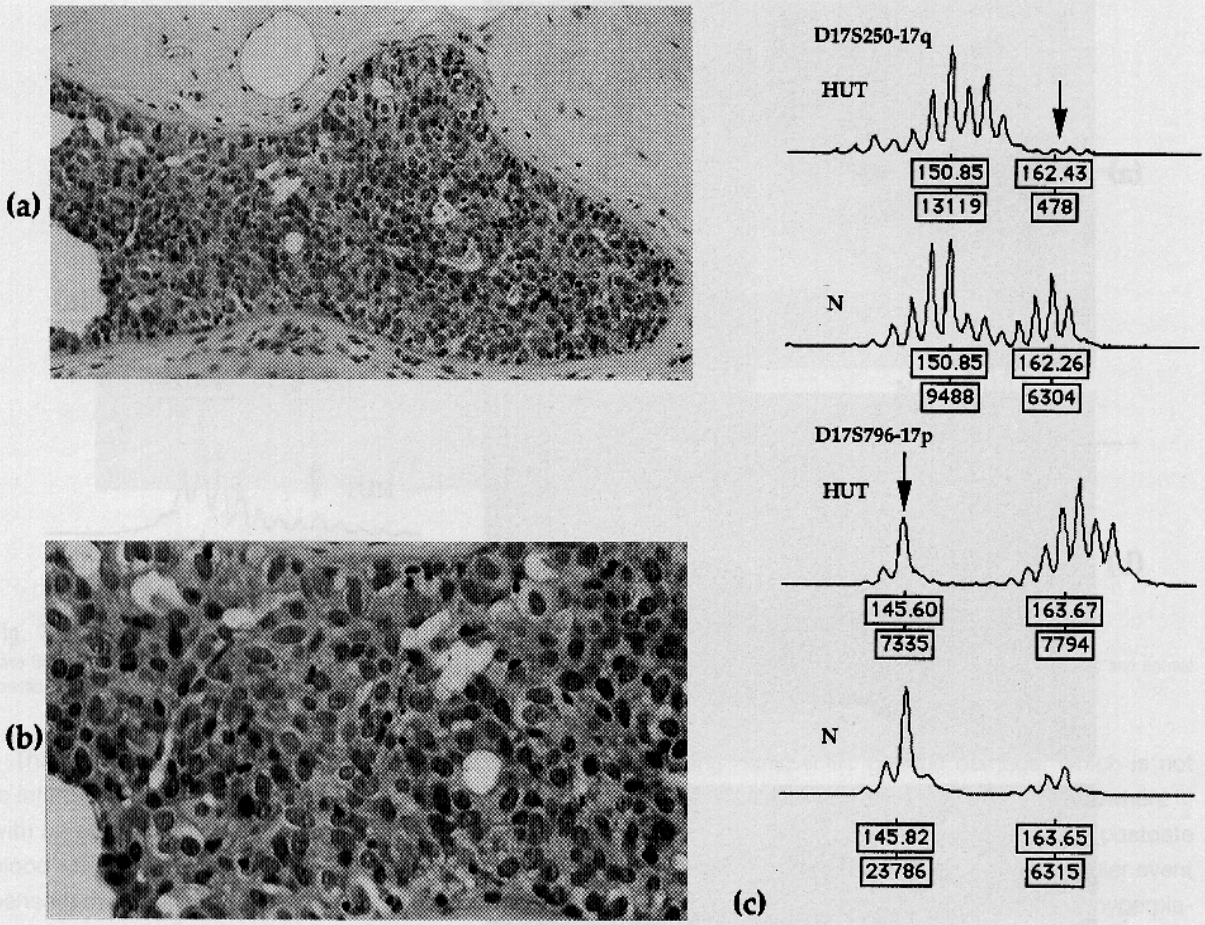

Fig. 1.
Case 34. (a), low power: HUT (hematoxylin and eosin × 160). (b), high power view of same lesion showing cytologic detail. There is morphologic polymorphism but no atypia (hematoxylin and eosin × 320). (c), AI at D17S250 (17q) and at D17S796 (17p) in the same sample. The bottom trace in each set is the normal alleles from the constitutional DNA. The figures below the alleles show allele size and AUC. The calculated allele ratios using the formula T1:T2/N1:N2 for 17q and 17p markers for HUT are 0.05 and 0.24, respectively. N = normal tissue.

We used very strict criteria for the assessment of AI, which was recorded only if the ratio of "tumor" to normal alleles was 0.25 or less and if it had been successfully reproduced in repeat experiments in a consistent fashion. There were a number of samples of hyperplasia in which more tentative evidence for AI was obtained. In these samples, it may be that the presence of contaminating DNA from myoepithelial, stromal, or leukocytes obscured the imbalance. Hence, the frequency of AI identified in hyperplasia may be an underestimate.

It is possible to determine AI by the method we have described only when the sequence amplified by PCR is lost from the great majority of cells (>75% because a cutoff ratio of 0.25 is used) in the sample. Where significant numbers of cells have retained the relevant allele, PCR will amplify it sufficiently to produce a second band and hence obscure the imbalance. The most likely explanation for the presence of AI in the cell population is that a majority of cells are descendants of a single cell in which the genetic lesion developed. The alternative explanation that all cells acquired the identical genetic lesion independently is highly improbable. This view is greatly strengthened by the finding in one case of AI at two loci in the same lesion. Consequently, our findings indicate that at least some HUT are monoclonal and, therefore, neoplastic rather than hyperplastic (polyclonal) proliferations.

We previously demonstrated AI at frequencies ranging from 11 to 55% in DCIS at loci on chromosomes 16q, 17p, and 17q. The frequency of AI in ADH was 25% at D17S796 (17p) and 55% at D16S413 (16q). LCIS showed a similar pattern to DCIS apart from the locus D17S796 on 17p, where the frequency of AI was much lower at 8%. The frequency of AI in HUT identified in the present study is much lower than that identified in *in situ* carcinoma or ADH. Although this may be a spurious difference resulting from cellular contamination as described above, the histologic identity of lesions with and without AI suggests that it is more likely to be a genuine finding. None of the AC or Pap exhibited AI. AC are circumscribed lesions with an orderly arrangement of uniform cells, which can be microdissected from the neighboring stroma with rel-

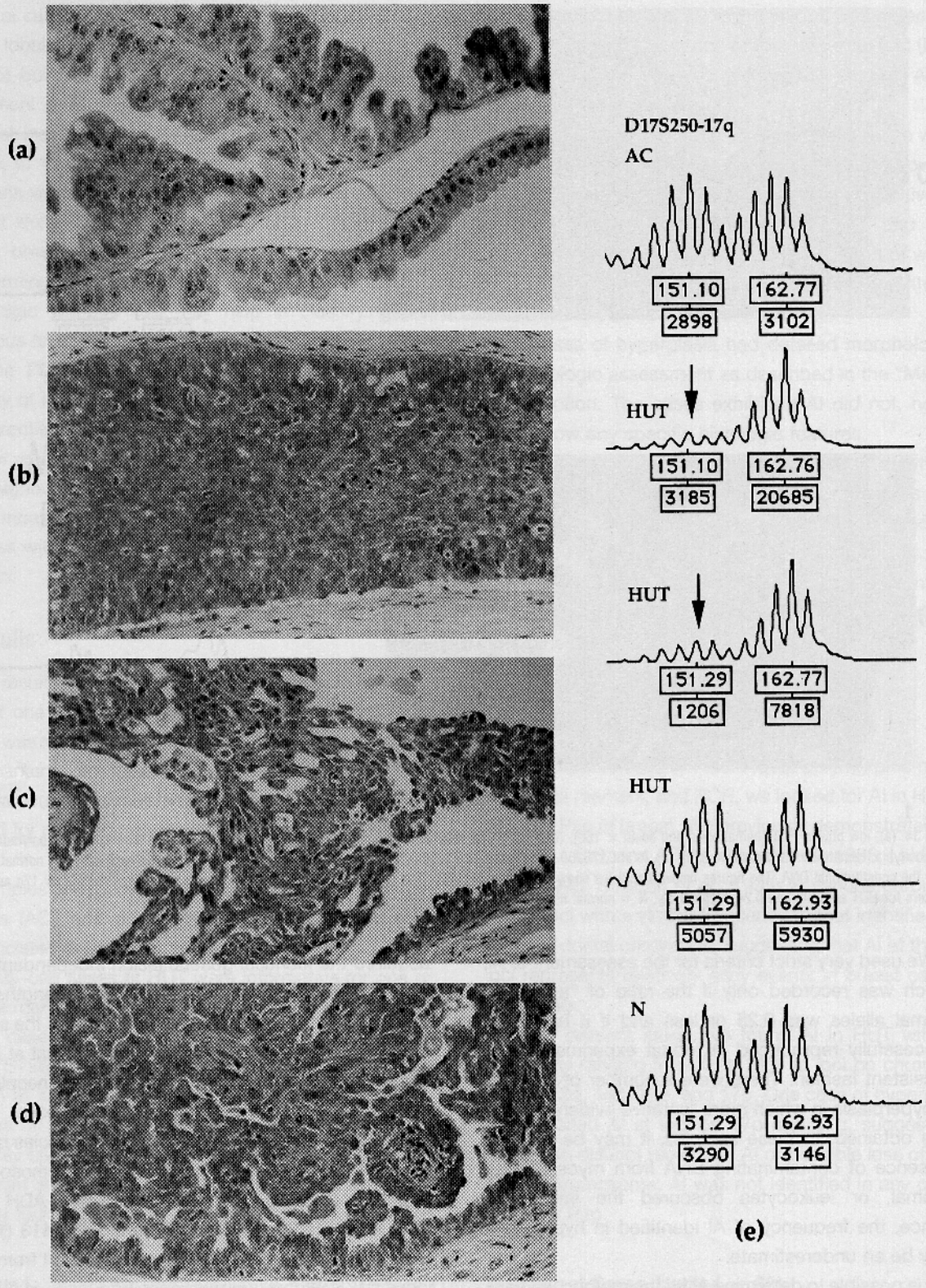

Fig. 2.

Case 46. (a), AC with single layer of monomorphic cells at bottom and papillary hyperplasia at top (hematoxylin and eosin × 320). (b-d), three foci of HUT from the same breast. The lesions exhibit varying degrees of proliferation. They are all morphologically polymorphic with irregular lumina and without atypia (all hematoxylin and eosin × 160). (e), data of allele ratios printed from Genotyper. The bottom trace shows the alleles from the normal constitutional DNA. The other four traces are of AC and the three HUT going from top to bottom. The two HUT shown in (b) and (c) exhibit AI at D17S250 (17q) (both ratios 0.15), but this is not demonstrated in either AC or HUT shown in (d). The figures below the alleles show allele size and AUC. N = normal tissue.

ative ease. Significant contamination from inflammatory or stromal cells is thus not likely to be the explanation for the lack of demonstrable AI. There appears to be a genuine difference, therefore, between HUT and AC; AI at the loci studied is either completely lacking in the latter or, less likely, is always restricted to subsets of cyst cells and consequently undetectable.

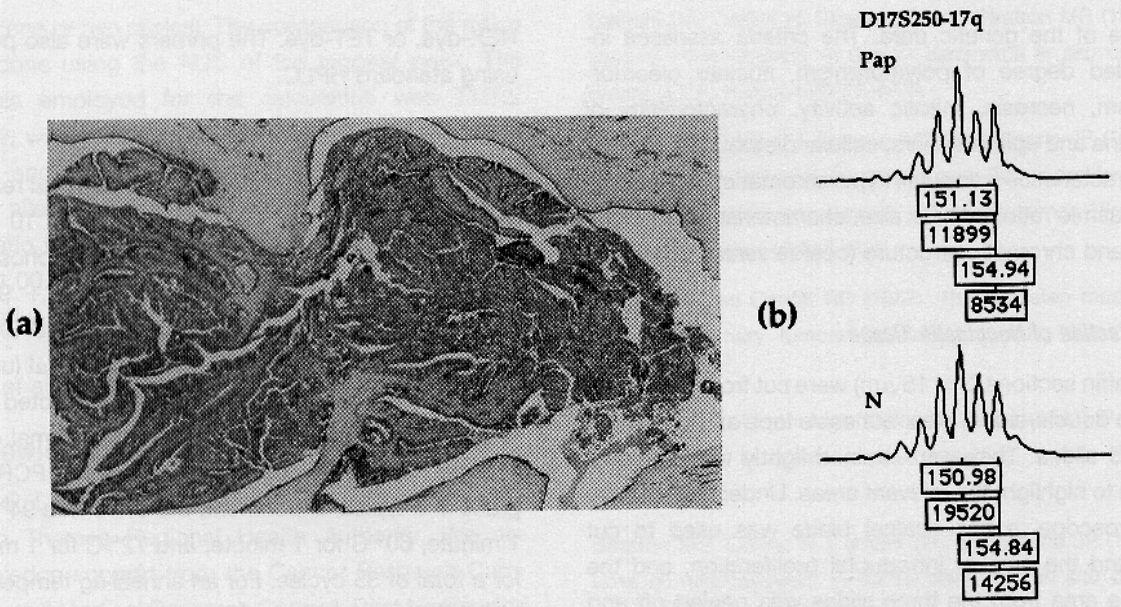

Fig. 3.
Case 68. (a), Pap of breast (hematoxylin and eosin × 160). (b), data from fluorescent analysis showing almost identical allele ratio (0.98) for Pap and normal constitutional DNA and hence no evidence of AI. N = normal tissue.

The lack of AI in Pap is interesting but more difficult to interpret. Pap are composed of fibrovascular cores with an epithelial lining. The cores also contain small blood vessels, and a myoepithelial layer is present beneath the luminal epithelial cells. Hence, the lack of LOH may be a result of the considerable difficulty of excluding a significant number of nonepithelial cells within the sample. The estimated contamination in these cases ranged from 40 to 50%. It has not been possible with present techniques to obtain only the luminal cells from these proliferations. Further studies employing interphase cytogenetics or comparative genomic hybridization or using DNA from sorted cells may resolve the problem. In addition, the possibility that Pap exhibit AI at polymorphic loci other than those studied cannot be excluded. The number of cases studied was also small. If AI in Pap is present in low frequency, a larger study would be required to demonstrate LOH.

Our findings show that at least some and perhaps all HUT are clonal and, therefore, neoplastic proliferations. As with hyperplastic alveolar nodules in the mouse mammary model (Cardiff and Muller, 1993), some "hyperplasias" in the human breast appear to represent benign neoplasms with limited growth potential and without obligation to transform into malignant tumors. It is not possible from the present data to draw any firm conclusions about a causal role for AI at these loci in HUT. If it is indeed pathogenetic, then the loci examined must be uninvolved in many cases. Alternatively, genetic changes affecting small chromosomal regions and thus not manifesting as AI may be responsible. AI may, however, be epiphenomenal, reflecting nonspecific genetic damage, which is not associated with causative genetic events elsewhere in the genome. A final possibility is that AI may postdate the onset of HUT and reflect an early molecular event in the progression to more atypical forms of hyperplasia or *in situ* carcinoma. The latter hypothesis would be consistent with the observation that HUT is associated with a significantly greater risk of developing carcinoma than AC (Dupont and Page, 1985). Large follow-up studies will be required before the significance of AI in preinvasive disease is clarified.

Methods

HUT, AC, and Pap were identified from benign breast biopsies. Criteria for the diagnosis were those adopted for the United Kingdom National Breast Screening Programme (Royal College of Pathologists Working Group, 1991).

A total of 68 benign breast biopsies suitable for study were identified. In 28 of these, more than 1 of the 3 lesions were studied; overall, these were 51 cases of HUT (87 lesions), 34 cases of AC (34 lesions), and 11 cases of Pap (12 lesions). Four cases of HUT and two cases of Pap had focal areas of apocrine metaplasia within them. In 17 of the 51 cases of HUT, more than 1 focus of hyperplasia was studied. In one case, two Pap were dissected from the same breast. All samples of AC, however, were from separate breasts.

All cases were initially identified and selected by SRL. The epithelial hyperplasias were characterized in detail by JPS, and this was done without prior knowl-

edge of the genetic data. The criteria assessed included degree of polymorphism, nuclear pleomorphism, necrosis, mitotic activity, characteristics of lumina and epithelial bars, cellular distribution, nuclear characteristics (vesicular/hyperchromatic), nuclear:cytoplasmic ratio, nuclear size, characteristics of nucleoli, and chromatin structure (coarse *versus* even).

Dissection of Neoplastic Tissue

Paraffin sections (3 × 15 μm) were cut from each case onto double-sided, clear adhesive tape and placed on glass slides. They were stained lightly with toluidine blue to highlight the relevant areas. Under a dissecting microscope, a fine scalpel blade was used to cut around the area of intraductal proliferation, and the same area from the three slides was peeled off and placed into a 1.5-ml eppendorf tube. Sections (5 μm) taken immediately before and after the 15-μm sections were stained with hematoxylin and eosin. These sections were used to identify and categorize the relevant lesions. Contamination in microdissected pieces was assessed by counting tumor and nontumor nuclei using an image analysis system.

DNA Extraction

Dissected fragments were incubated for 16 hours at 37° C in 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 1% (w/v) SDS, and 500 μg/ml proteinase K. The mixture was then heated to 100° C for 10 minutes to inactivate the proteinase K, and aliquots were used directly in the PCR.

AI was investigated by amplification of polymorphic microsatellite markers using fluorescence-tagged primers and PCR. The PCR products were analyzed using ABI 373A automated fluorescent DNA sequencer (Applied Biosystems, Foster City, California) and were automatically analyzed by the 672 GeneScan Analysis Software (version 1.2.2–1) (Applied Biosystems) at the end of the run.

Primers

Four dinucleotide repeats were used for this study. These are D13S267 (13q), D16S413 (16q), D17S796 (17p), and D17S250 (17q). These lie within genomic regions, which exhibit LOH in IC. D17S796 lies in the vicinity of the p53 gene, D17S250 in the region of the BRCA1 gene, and D13S267 in the region of the BRCA2 gene. All of the markers apart from D17S250 (Weber *et al.*, 1990) were identified from the second-generation linkage map constructed by the Genethon group (Weissenbach *et al.*, 1992). One primer of each pair was fluorescently labeled using either FAM-dye, HEX-dye, or TET-dye. The primers were also purified using standard HPLC.

PCR

The DNA was amplified by PCR using a 10-μl reaction mixture. The PCR mix consisted of 1 μl of 10 × taq polymerase buffer, 1 μl dinucleoside 5′-triphosphate (2 mM each nucleotide: 20 μl nucleotide + 920 μl water), 0.25 μl (10 mg/ml) BSA, 0.38 μl (1 OD U/ml) of each primer, 0.02 μl Taq polymerase, 2.35 μl (up to 5 μl) of water, and 4 μl of paraffin-extracted DNA template. The DNA was amplified in a thermal cycler (Hybaid, Omnigene) using a touch-down PCR program. In a typical experiment, the cycle was 94° C for 1 minute, 60° C for 1 minute, and 72° C for 1 minute, for a total of 35 cycles. For an annealing temperature of 60° C, the program was set at a temperature of 70° C with a stepwise reduction by 2° C until 60° C was reached.

Electrophoresis

The PCR products were analyzed on 6% polyacrylamide denaturing gels (prepared using BioRad reagents) in 1 × Tris-borate EDTA buffer using an ABI 373A automated fluorescent DNA sequencer (Applied Biosystems), which has a four-color detection system. Two microliters of each PCR reaction was combined with 4 μl formamide and 0.5 μl of a TAMRA fluorescent size marker (GS500, Applied Biosystems). This mix was denatured for 4 minutes at 94° C, after which 4 μl was loaded into each well on a prewarmed gel. The gel was run for 5 hours at 30 watts, 20 amps, 1300 volts, and 40° C. While the samples were undergoing electrophoresis, the fluorescence was detected in the laser scanning region using filter set B and was collected and stored using the 672 GeneScan Collection Software (Applied Biosystems).

Data Analysis

The fluorescent gel data collected during the run were automatically analyzed by the 672 GeneScan Analysis Software (version 1.2.2–1) (Applied Biosystems) at the end of the run. Each fluorescent peak was quantitated in terms of size (in base pairs), peak height, and peak area. The results were then imported into Genotyper (version 1.1) (Applied Biosystems) for further analysis and printing. Genotyper was set up to list the size, peak height, and peak area for each allele, thus allowing quantitative comparison among the alleles and facilitating the calculation of allele ratios.

Assessment of AI by Calculating Allele Ratios

The normal control samples were used to assess whether the sample was homozygous or heterozy-

gous (one or two alleles). The comparison of the ratios was done using the AUC of the biggest peak. The formula employed for the calculation was T1:T2/N1:N2, where T1 and N1 are the AUC of the smaller allele, and T2 and N2 are the relevant figures for the larger allele. For ratios greater than 1, the reciprocal of the ratio is calculated to give a figure between 0.00 and 1.00. We assigned a value of 0.25 or less as indicative of AI.

Date of acceptance: To come

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