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Short Communication

Loss of Heterozygosity and Microsatellite Instability in Breast Hyperplasia

No Obligate Correlation of These Genetic Alterations with Subsequent Malignancy

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Loss of heterozygosity and microsatellite instability have been often reported in breast cancer and seldom in proliferative breast disease (PBD). DNA samples from microdissected PBD lesions, including papillomas (25 lesions), from 8 women were analyzed by polymerase chain reaction for loss of heterozygosity and microsatellite instability at 10 loci including INT-2 oncogene locus, D17S796 (the p53 gene region), and D17S579 (in the region of the BRCA-1 gene). In a patient, five loci with microsatellite instability and two loci with loss of heterozygosity were identified in one papilloma with florid hyperplasia and atypia, and 10 other PBD lesions were negative for genetic alteration (GA) and atypia. Three loci with microsatellite instability were identified in another PBD lesion without atypia, whereas another lesion from this second patient had minimal atypia without GAs. These two patients have been well for more than 20 years. No other patient, including a woman developing cancer, had GAs. We detected GAs in PBD (25% of women, 8% of lesions). Incomplete correlation between GAs and anatomic atypia was suggested. It seems evident that several GAs in PBD lesions may not indicate clinically meaningful premalignancy for remaining breast. (Am J Pathol 1997, 150:1925–1932)

Breast carcinoma is the most frequent non-skin cancer of women in much of the world.[1,2] Multiple genetic alterations have been reported in *in situ* and invasive breast cancer and, rarely, in benign proliferative breast disease.[3–10] In both breast and colon cancer, there is strong evidence of a progression of molecular abnormalities as lesions evolve from premalignant disease to invasive cancer.[11] Histological and cytological atypia are also known to provide anatomic evidence that is also predictive of malignancy.[12] Many studies have found hyperplastic lesions to be predictive of increased cancer risk. Florid hyperplasia without atypia is associated with a cancer risk approaching double that of otherwise similar women. Specifically defined patterns of atypia by histology are predictive of a four to five times elevated cancer risk, a risk of cancer that is intermediate between ductal carcinoma *in situ* (DCIS) and hyperplasias without atypia.[13–15]

The concurrence or lack thereof of genetic alterations (GAs) in anatomically defined benign and premalignant breast lesions has been little studied[5,7,10]; either anatomic alterations or GAs can occur without immediate malignant implication. Certainly, many al-

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Table 1. *Histology, Follow-Up, and Results*

Case	Age (years)	Sex	HP studied	PAP studied	Clinical data, follow-up	Result
1	83	F	0	2		No MSI or LOH
2	65	F	0	3		No MSI or LOH
3	67	F	0	1		No MSI or LOH
4	42	F	3	0	Alive without cancer for 21 years	MSI in one FH without atypia
5	31	F	1	0	Breast cancer 13 years later; alive for 8 years	No MSI or LOH
6	17	F	6	6	Alive without cancer for 25 years	MSI and LOH in one FH with atypia
7	39	F	1	0	Malignant melanoma at the time of biopsy	No MSI or LOH
8	47	F	2	0	Alive without cancer for 21 years	No MSI or LOH

HP, hyperplasia; PAP, papilloma, or micropapilloma (with or without included hyperplasia); F, female; FH, florid hyperplasia.

terations, including chromosomal changes, occur in fully developed malignancy defined by anatomic criteria. O'Connel et al[16] have reported that 63% of proliferative lesions in conjunction with cancer showed at least one locus of loss of heterozygosity (LOH). Lakhani et al[5] found that 50% of 10 informative cases with atypical ductal hyperplasia (some of which were not associated with cancer) showed at least one locus of LOH.[5]

Microsatellite instability (MSI) has been reported as a very early event in tumorigenesis of many organs, including breast.[17,18] The role of MSI remains controversial,[19,20] but it is likely a precursor event to neoplastic transformation in some cells.[21]

We have studied the surgical biopsy specimens obtained from eight women with benign breast disease and examined LOH and MSI in hyperplastic lesions using dinucleotide repeats at 10 loci that have been frequently reported GA sites in breast cancer.[4,5,16,22,23] We seek to answer the following questions. 1) Does the finding of LOH and MSI in a breast biopsy usually indicate future evolution of malignant lesions? 2) Are such GAs present in hyperplasia with concomitant histological atypia, or may these GAs occur in banal lesions?

Materials and Methods

Cases and Tissue

The breast biopsies we studied were from a large cohort of women undergoing surgery in Nashville, TN.[15] The examined cases are listed in Table 1. Eight serial sections were cut from blocks of paraffin-embedded, formalin-fixed tissue. We microdissected several lesions in each biopsy when the size was more than 1 mm and when the lesion was detected in several serial slides.

The first, fourth, and last sections were hematoxylin and eosin (H&E) stained as a template to guide the microdissection of the serial 10-μm unstained sections. A lesion was outlined by a 27-gauge needle with a microscope, and then the dust was taken off by air. The isolated lesion was dissected using a surgical blade. The needle was cleaned by bleach and 80% ethyl alcohol. Each lesion and the adjacent normal tissue was independently scraped, and blades were changed after each microdissection. The scraped tissue was transferred to a microfuge tube, and the identity of the tissue removed was verified by microscopic analysis of the remaining tissue on the glass slide.

DNA Extraction

DNA was extracted from paraffin as described by Sukpanichnant et al[24] with no modifications. Sections were deparaffinized in graded alcohols, incubated at 55°C overnight in digestion buffer (50 mmol/L Tris (pH 8.3), 1 mmol/L EDTA, 0.5% Tween-20) with 200 μg/ml proteinase K, and heated for 5 minutes at 100°C. Residual paraffin was pelleted by centrifugation, and varying amounts of lysate were used in the polymerase chain reaction (PCR) amplification. Additional DNA was extracted from a peripheral blood sample of a female co-author for positive control as described by Kunkel.[25]

PCR Analysis

Nine primer pairs for the microsatellite loci were synthesized at the Vanderbilt University School of Medicine, Department of Molecular Physiology. The 10th pair, INT-2, was obtained from Research Genetics, (Huntsville, AL). The microsatellites studied consisted of seven CA and three GT dinucleotide repeats, and their heterozygosity indices were more than 80%. The location of the seven CA repeats were D1S243 (1p), D2S172 (2q33–q37), D6S311 (6q21–q23.3), D8S256 (8q24.13-qter), D11S934 (11q23.3–q24), D14S81 (14q31), and D17S579 (in the region of the BRCA-1 gene).[26] The location of the three GT

Table 2. *Correlation between Microscopic Atypia and Genetic Alterations*

Histological diagnosis	Number of cases	Number of lesion(s)	GAs
Multiple PAP & HP & focal atypia	1	1 HP & PAP & atypia	5 MSI and 2 LOH
		11 HP or PAP usual	None
HP & focal mild atypia	1	1 HP usual	3 MSI
		1 HP with atypia	None
		1 HP usual	None
PAP or hyperplastic lesion without atypia	6	10	None

HP, hyperplasia; PAP, papilloma or micropapilloma.

repeats were D16S413 (16q24.3), D17S796 (in the p53 gene), and the INT-2 oncogene locus (11q13).[17,27] All of the microsatellite markers described were studied using the following standard PCR conditions. One primer of each pair was labeled at its 5′ end by using [^{32}P]ATP and T4 polynucleotide kinase. PCR assays were performed in 50 μl of 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 1.5 mmol/L $MgCL_2$, 2.5 U of *Taq* polymerase, 2.5 mmol/L each dNTP, 5 to 50 μmol/L primer, and 1 to 6 μl of DNA. After a first denaturation step of 5 minutes at 95°C, amplification was carried out during 35 cycles of 94°C for 40 seconds, 55°C for 30 seconds, and 72°C for 1 minute. A positive control reaction containing DNA from peripheral blood and a negative control reaction containing no DNA were examined for each PCR assay. DNA amplification was stopped by adding 7 μl of the gel loading solution (95% formamide, 10 mmol/L EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). The PCR products were denatured for 3 minutes at 95°C before loading 15 μl onto a 6% denaturing polyacrylamide gel. Autoradiography was for 30 minutes to 1 week at −70°C. Cases showing positive alterations were repeated for confirmation at least twice. Alterations were independently scored without knowledge of histological type or clinical details.

Results

Most of these eight women studied had long-term clinical follow-up so that clinical outcomes were available. Table 1 displays the cases studied.

We examined 25 hyperplastic lesions and/or micropapillomas found in eight breast biopsies from eight different women. Each of these samples was evaluated for 10 microsatellite repeat loci. When negative control reactions failed to generate products and positive control reactions successfully amplified, the results from that assay were scored. The DNA of normal tissue from each woman was simultaneously analyzed. In comparison with the normal, GAs were detected in a single lesion in two of eight women (25%). The other lesions in these two women and all lesions of the six other women were scored negative for LOH or MSI at the loci tested (Table 2). In case 6, one lesion of florid hyperplasia with atypia had demonstrated LOH at two loci (INT-2 and D11S934; Figure 1 and Table 3) and MSI at five loci (D1S243, D2S172, D6S311, D8S256, and D14S81; Figure 1 and Table 4). In case 4, one hyperplastic lesion without atypia had MSI identified at three loci (D6S311, D8S256, and D17S796; Figure 2 and Table 4). Because one case was homozygous and uninformative for each of the two loci (INT-2 or D11S934) for LOH detection, LOH occurred at a frequency of 1/7

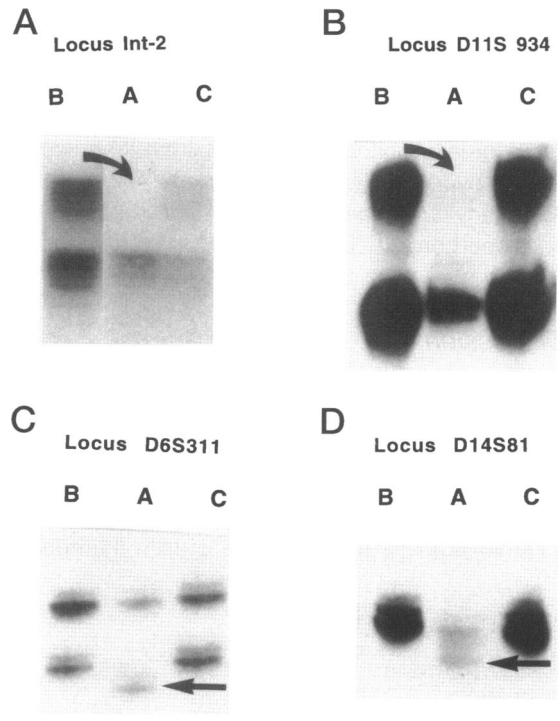

Figure 1. *LOH in case 6. Allelic losses are indicated by* arrows *on two loci: INT-2* (A) *and D11S934* (B). *Both LOH are detected in the lesion of florid hyperplasia with atypia* (*see Figure 3, a and b*). *Examples of MSI in case 6 were observed at loci D6S311* (C) *and D14S81* (D). *Shifted alleles are indicated by* arrows. *MSI is detected in the same lesion positive for LOH.* Lane A, *hyperplasia with atypia;* lanes B (*Figure 3c*) *and* C, *hyperplastic lesions of the same case and without atypia. The electrophoretic mobility of DNA from normal tissue is the same pattern as in* Lanes B *and* C (*normal data not shown*).

Table 3. *LOH*

Case	D1S243	D2S172	D6S311	D8S256	INT-2	D11S934	D14S81	D17S796	D17S579	D16S413
1	0/2	HO	0/2	HO	0/2	0/2	0/2	0/2	0/2	HO
2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	HO
3	0/1	0/1	HO	0/1	0/1	HO	0/1	HO	0/1	0/1
4	0/3	HO	HO	0/3	HO	0/3	0/3	0/2	0/3	0/3
5	0/1	HO	HO	0/1	0/1	0/1	0/1	0/1	0/1	0/1
6	0/12	0/11	0/11	0/12	**1/12**	**1/10**	HO	0/12	HO	HO
7	0/1	–	HO	0/1	0/1	0/1	HO	HO	0/1	0/1
8	0/2	0/1	0/1	HO	0/1	0/2	HO	HO	0/2	0/2

Results are presented as positive lesion (bold) per lesions studied in the same case. HO, homozygous case; –, result not obtained.

(14%; Table 3). MSI occurred at a frequency of 2/8 (25%). The loci of D6S311 and D8S256 showed MSI in two of these cases. No GAs were found for the primers D17S579 or D16S413.

LOH and MSI were only detected in the single lesion with histological atypia (Figure 3, a and b). The lesion showed atypia similar to that reported by us in multiple micropapillomas.[28] The other 11 lesions of case 6 had neither GAs nor histological atypias (Figure 3c). In case 4, there were no histological differences between the lesions with GAs (Figure 3, d and e) and one of the other two lesions that lacked GAs. Both lesions were without histological atypia. However, a third lesion in this biopsy had a recognizable atypia reflected in cytology and histological patterns, ie, mild atypia (Figure 3f). This mildly atypical lesion lacked full criteria for atypical ductal hyperplasia but had a greater uniformity of cell pattern than usually seen in hyperplasias without atypia.[29,30] We did not detect any GAs in a single patient who subsequently developed breast cancer (case 5).

Discussion

Many GAs have been described in malignant neoplasms. There is a tendency for these to be increased in number in cancers more likely to be clinically malignant.[11,31,32] Indeed, many of these alterations may actually be secondary to the carcinogenic process and therefore of little therapeutic use and no promise for early prevention or diagnosis strategies.[33]

It is because biologically advanced lesions have multiple genetic alterations that there has been a concerted effort to look for the earliest changes. It is hoped that specific changes will be identified before the phenotype evolves to represent a threat to life and therefore provide opportunities available for preventive, therapeutic, and diagnostic strategies. Many studies have targeted these attempts to find early changes in noninvasive lesions in breast cancer as well as atypical hyperplastic lesions.[5,7,10]

It is in this context that we have endeavored to look at hyperplastic lesions present in individuals with no known association with any other known increased risk of breast carcinoma. We evaluated 25 hyperplastic lesions for LOH and MSI at 10 regions of the genome in which such GAs have been described in mammary carcinoma.[16,22,23,27,34,35]

We found five MSIs (Table 4) and LOH for INT-2 (11q13) and D11S934 (11q23.3–24) (Table 3) in a case. Deletion of chromosome 11q might have occurred in this lesion because these two loci in 11q are widely separated. Although LOH and amplification of 11q have been reported in various cancers,[3,16,22,23,35–39] this is the first report of LOH for INT-2 and D11S934 in premalignant lesions. Also, this is the first report that has detected multiple MSIs in premalignant lesions and no association with cancer. MSI is a landmark for some sporadic and he-

Table 4. *MSI*

Case	D1S243	D2S172	D6S311	D8S256	INT-2	D11S934	D14S81	D17S796	D17S579	D16S413
1	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
3	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
4	0/3	–	**1/3**	**1/3**	0/3	0/3	0/3	**1/2**	0/3	0/3
5	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
6	**1/12**	**1/11**	**1/11**	**1/12**	0/12	0/10	**1/12**	0/12	0/11	0/10
7	0/1	–	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
8	0/2	0/1	0/1	0/2	0/1	0/2	0/2	0/2	0/2	0/2

Results are presented as positive lesion (bold) per lesions studied in the same case. –, result not obtained.

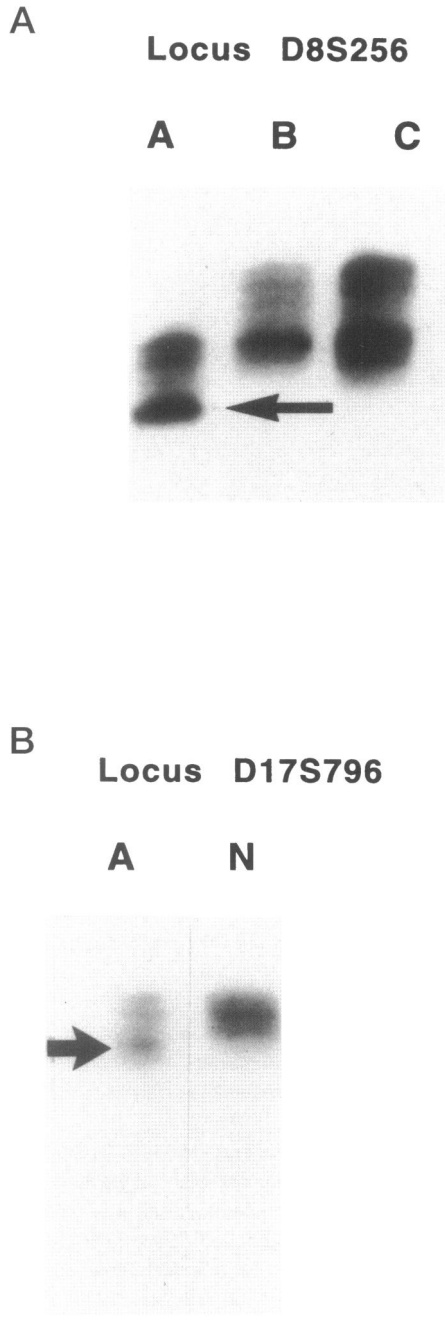

Figure 2. *MSI in case 4 on loci D8S256* (A) *and D17S796* (B). *Shifted alleles are indicated by* arrows. *The lesion positive for MSI* (A) *is hyperplastic lesion (HL) without atypia (Figure 3, d and e).* Lane B, *HL without atypia;* lane C, *the other HL with mild atypia (*Figure 3f); N, *normal tissue. The electrophoretic mobility of DNA from normal tissue is the same pattern as in* Lanes B *and* C.

reditary cancers of the colon and may be due to a defect in replication or repair.[40,41] Inactivation of the type II transforming growth factor-β (a potent inhibitor of epithelial cell growth) has been reported in colon cancer cells with MSI.[42] Deficient DNA repair capacity has been reported in women with breast cancer.[43] But MSI is not necessary for carcinogenesis.[19]

Although multiple GAs were detected in this case, the patient has been alive and free of breast cancer for more than 25 years (Table 1). The other patient who developed cancer 13 years after the biopsy had no GAs detected. However, it is likely that some loci of LOH and/or MSI may have implications for later development of malignancy.[44] Despite the fact that current studies are few, certainly not all GAs lead to cancer. At least a subset of hyperplasias may have LOH and/or MSI and do not indicate a meaningful alteration and risk of cancer, at least when the lesion is surgically removed. This has always been a limitation of such studies of premalignancy in humans when removal of a lesion is necessary for its detection.[45]

Usually the presence of somatic mutations has been associated with histologically advanced patterns of atypical hyperplasia and DCIS. Rarely, GAs have been found in hyperplastic lesions without anatomic atypia. Lakhani et al[10] have recently reported that several cases exhibiting GAs did not show any specific hyperplastic, anatomic features, whereas Lizard-Nacol et al[46] found none. We detected GAs in a hyperplastic lesion with atypia (Figure 3, a and b) but not in the other 11 lesions without atypia (Figure 3c) in case 6. Also, we detect GAs in a hyperplastic lesion without atypia (Figure 3, d and e) but not in the other lesion with mild atypia (Figure 3f) in case 4. Our results suggest that GAs are more likely to occur in patients with histological atypia in hyperplastic lesions although not always in the same lesion (Tables 1 and 2). We conclude that close correlation between histological and genetic changes are found but imperfectly so. Histological and molecular changes may not necessarily be precisely correlated. Additional investigation of these associations is clearly required. However, the association of GA in lesions with histological atypia links these somatic mutations to factors that have been shown to denote increased cancer risk in epidemiological studies.

The significance of these changes may be most profitably estimated by statistical analysis of biological association as noted by Callahan and Campbell[34]: "Many have sought to characterize somatic mutations that are either selected or develop early in the progression to malignancy in solid organs,. . .but future statistical analysis may be more informative by considering the association between groups of specific mutation and outcome." Certainly, it is clear that increasing anatomic (cytological and histological patterns) atypia increases the likelihood of finding GAs. This is shown with the presence of most alter-

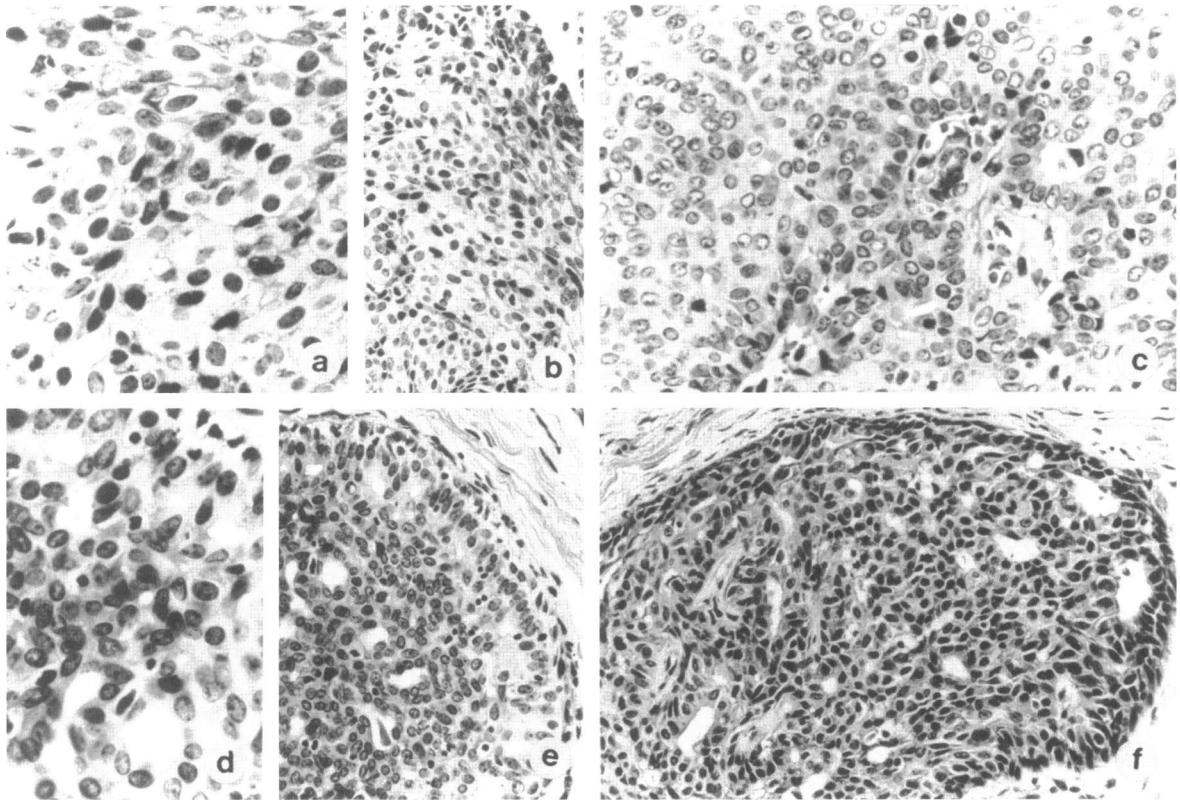

Figure 3. *H&E.* **a** *and* **b**: *Florid hyperplasia with atypia. The cells are enlarged with somewhat clear cytoplasm and regularly oval nuclei in high-power view* (**a**). *The uniformity of cytology and cell placement establishes atypia in this setting. We found multiple GAs in this lesion.* **c**: *Hyperplastic lesion (HL) without atypia. This is one of the microdissected lesions of HL without atypia. Nuclei are hypochromatic, heterogeneous, and smaller than in* **a**. *Fibrovascular cores represent micropapilloma. Neither LOH nor MSI were found in this lesion or the other similar lesions from the same biopsy.* **d** *and* **e**: *HL without atypia. Many irregular spaces between cells and mild nuclear variability deny an assignment of atypia. In a solid cellular area in high-power view* (**d**), *the uneven placement of the cells is evident and denies features of atypia.* **d** *and* **e** *show the lesion positive for MSIs.* **f**: *HL with mild atypia. Hyperchromasia and irregular cell placement are seen.* **f** *is a lesion negative for GAs. Magnification,* ×*60* (**a**, **c**, *and* **d**) *and* ×*30* (**b**, **e**, *and* **f**).

ations of p53 and erb-2 in only high-grade DCIS and their absence in atypias.[47,48] The co-occurrence of GAs and mildly atypical hyperplastic lesions is obviously rare and may have different implications for premalignancy than when found in more advanced lesions, recognized as DCIS.

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