Detection of Monoclonal Microsatellite Alterations in Atypical Breast Hyperplasia

Carol L. Rosenberg,*§ Antonio de las Morenas,‡ Katie Huang,* L. Adrienne Cupples,‖ Douglas V. Faller,*§ and Pamela S. Larson*

**Cancer Research Center, and the Departments of ‡Pathology and §Medicine, Boston University School of Medicine, and the ‖Department of Epidemiology and Biostatistics, Boston University School of Public Health, Boston, Massachusetts 02118*

Abstract

Atypical hyperplastic (AH) breast lesions are currently classified and treated as benign proliferative disorders, but their presence is associated with a four- to fivefold increased risk of developing breast cancer. Currently, it is not known if an AH lesion is a marker of increased risk, or is itself a premalignant lesion. To investigate this question, we used a series of 15 microsatellite loci to analyze 15 separate AH lesions microdissected from the archived pathology specimens of subjects with no coincident or previous breast malignancy. We found that a significant subset (6/15, or 40%) of these AH lesions demonstrated evidence of monoclonal microsatellite alterations, both length variation and allele loss. These monoclonal alterations suggest that the AH lesion has already undergone genetic changes conferring a growth advantage. Thus, these AH lesions may actually be early neoplasms. We also noted that monoclonality characterized AH lesions in younger as compared with older women (44 vs. 59 yrs, $P < 0.05$) and that a subset of monoclonal lesions (4/6) demonstrated microsatellite alterations at more than one locus, suggesting that an undetermined type of genetic instability may play a role early in the development of abnormal breast proliferations. These findings contribute to our understanding of the pathogenesis of AH lesions and may have implications regarding their relationship to breast tumors. (*J. Clin. Invest.* 1996. 98:1095–1100.) Key words: proliferative breast disease • instability • clonality • premalignant • neoplasm

Introduction

Increased incidence and awareness of breast cancer have led to increased screening for malignancy, which has resulted in the identification of an increased number of suspicious lesions, many of which are biopsied. Approximately 90% of biopsies reveal no malignancy. Some are normal and many are diagnosed as various types of "benign" breast disorders. Benign, however, is not the same as normal, and epidemiologic studies demonstrate that certain histologic types of benign breast disorders are associated with a significant increase in the risk of developing breast cancer (1–4). For example, hyperplastic (or proliferative) lesions without atypia are associated with an approximately twofold increased risk, and atypical hyperplastic (AH)[1] lesions with a four- to fivefold increased risk. It is not known if high risk proliferative lesions, such as AH, are simply markers of increased risk, or could themselves be actual precursors of malignancies.

To address this issue, we chose to investigate whether genetic abnormalities could be detected in benign proliferative lesions. Such a finding would implicate these lesions as potential precursors of breast cancers and could shed light on genetic abnormalities that play a role early in breast tumorigenesis. We have investigated whether AH lesions demonstrate evidence of monoclonal alterations of microsatellite sequences. Monoclonality, a fundamental characteristic of neoplasia, in general implies that a single cell, from which the monoclonal population has arisen has undergone changes that have been selected for, perhaps by conferring some growth advantage to the cell. To determine whether monoclonal, genetically abnormal cell populations exist within AH lesions, we used a PCR-based technique to identify alterations in di-, tri-, or tetranucleotide repeat sequences, known as microsatellites, in DNA extracted from microdissected AH lesions, in comparison with normal breast tissues. The frequency of spontaneous dinucleotide microsatellite length variation in normal cells is believed to be low (< 0.5%) (5), as is the "background" rate of detecting an isolated microsatellite alteration in malignant tissue (0.7%) (6). Frequent microsatellite alterations, thought to be a manifestation of defective DNA replication and repair, were first noted in familial colon cancers (7–9). Less frequent alterations are also detectable in a subset of breast cancers (10–16). Regardless of its frequency or etiology, a microsatellite alteration (either length variation or allele loss) that occurs in a cell that does not also undergo clonal expansion should not be detected among the large excess of normal DNA from surrounding cells.

We have investigated AH lesions from archival breast biopsy specimens containing neither in situ nor invasive carcinoma. Using a panel of PCR primers amplifying 15 microsatellite loci from 12 different chromosome arms, we examined DNA from these microdissected AH lesions for evidence of

Address correspondence to Dr. Carol L. Rosenberg, Boston University Medical Center, 80 E. Concord St. R908, Boston, MA 02118. FAX: 617-638-5609; E-mail: crosenberg@med-med1.bu.edu

Received for publication 28 March 1996 and accepted in revised form 26 June 1996.

J. Clin. Invest.

0021-9738/96/09/1095/06 $2.00
Volume 98, Number 5, September 1996, 1095–1100

1. *Abbreviations used in this paper:* AH, atypical hyperplasia; LOH, loss of heterozygosity.

length variation or allelic loss. We find that a subset of AH lesions, particularly those in younger women, contains monoclonal, genetically abnormal populations of cells.

Methods

Selection of samples. All available Boston City Hospital and Boston University Medical Center Hospital Pathology Department records of breast biopsies performed between 1986 and mid-1995 were examined to identify specimens containing one or more AH lesions but neither carcinoma in situ nor invasive carcinoma. 62 reports were identified but nearly one-half of these cases was eliminated from study because no slides or tissue blocks were available, or because technical difficulties with the slides or fixatives made reliable diagnosis impossible. In a number of cases, the lesions did not contain enough cells to yield sufficient DNA for analysis. From the remaining specimens, the original or a newly prepared slide was reviewed by a single breast pathologist. All evaluable specimens were of ductal histology. The term atypical ductal hyperplasia was used when either cytological or architectural criteria of ductal carcinoma in situ were met, but both were not present completely at the same time (17). In 12 cases, the original diagnosis of AH was not consistent with currently accepted diagnostic criteria. For each confirmed case, seven serial sections were cut from the block, the top and bottom sections were stained with hematoxylin and eosin, and each was examined to reconfirm the diagnosis of AH in both slides. In five cases, the AH lesion was not present in the bottom slide, or changed histologic appearance. These cases were not included in this study, leaving 14 evaluable specimens.

Microdissection. For each case, the two hematoxylin and eosin–stained slides were used as guides to locate the AH lesion(s) which was then microdissected away from the surrounding tissue with a sterile 22-gauge needle, under a light microscope. As control tissue for each case, normal appearing duct and, if available, stromal tissue from the same biopsy were also microdissected. Control tissues and corresponding AH lesions came from the same biopsy specimen, and usually the same tissue block. Thus, they had been fixed, handled, and stored identically. Each specimen was estimated to contain between 250 and 1,000 cells.

Preparation of DNA. DNA was isolated from each sample using a method described previously (18). In two cases, the DNA was found to be too degraded for analysis.

PCR. 15 sets of microsatellite primers (see Table I) were used in a series of multiplex polymerase chain reactions. Primers for the AR and D18S34 loci were synthesized (model 392A synthesizer; Applied Biosystems, Inc., Foster City, CA). All other primers were purchased from Research Genetics (Huntsville, AL). 2–3 μl of extracted DNA was used in a 50-μl multiplex polymerase chain reaction that also contained between 6 and 16 pmol of each primer; a standard PCR reaction buffer (GIBCO-BRL, Gaithersburg, MD) plus 1.5 mM $MgCl_2$; 200 μM of each dATP, dGTP, and dTTP, and 20 μM of dCTP; and 0.2 μl of [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA). Reactions were heated to 95°C for 5 min, then cooled to 80°C and 1 U of *Taq* polymerase (GIBCO-BRL) was added. 40 cycles of amplification were carried out using cycling parameters of 95°C for 1 min, 55, 58, or 60°C for 1 min, 72°C for 1 min, with a final extension of 10 min. 2–6-μl aliquots were removed, mixed with 2 μl of loading dye, and heated to 95°C for 10 min before being chilled on ice, followed by loading onto 7% polyacrylamide, 5.6 M urea, 32% formamide gels and separated at 80 W for 3.5–4 h. After drying, gels were exposed to film for 2 h to 3 d. To confirm results and to eliminate the possibility of PCR artifact, all reactions from each subject were repeated under the same conditions and run on adjacent lanes of the same gel at least twice.

Clinical data. Clinical information was obtained from existing medical records and recorded in such a way that subjects could not be identified directly or through identifiers linked to the subjects.

Statistical analysis. The mean ages of those identified as monoclonal were compared with those who had no evidence of monoclonality by means of Student's *t* test. Comparison of family and personal histories between the two groups were evaluated by a χ^2 test.

Results

15 microsatellite loci were selected according to one or more of the following criteria: (*a*) location at sites of tumor suppressor genes or regions reported to demonstrate loss of heterozygosity (LOH) in early breast cancers; (*b*) loci reported to demonstrate length variation in breast or other cancers (including sites near some of the mismatch repair genes); (*c*) dispersion throughout the genome; (*d*) inclusion of di, tri, and tetra nucleotide repeats; (*e*) high heterozygosity (since the amount of DNA available is so limited, a locus that is informative only 50% of the time is not an optimal choice); and (*f*) amplification of a relatively small product (i.e., $\leq$ 200 bp to assure reproducible results from the fragmented, fixed DNA). Table I lists the microsatellites studied. We examined DNA extracted from 15 microdissected AH lesions derived from 12 subjects without previous or coexisting breast cancer, and compared it with DNA extracted from each subject's normal appearing breast duct(s) and, if available, breast stromal tissue. In one instance (subject 23) a hyperplastic lesion without atypia (H) was also available for comparison. An average of 13 loci yielded information for each lesion. Occasionally, as others have also noted, larger sized fragments (for instance, at the D2S123 locus) could not be amplified consistently, presumably because of degradation of the fixed DNA; in addition, subjects were usually homozygous at one or two loci. Because the amount of DNA extractable from the microdissected lesions is too small to allow quantitation, comparison of relative band intensities would not be accurate. Therefore, only substantial gains or near-total losses were scored as evidence of microsatellite locus alteration. This may lead to an underestimation of the incidence of monoclonality.

We found that 6/15 (40%) AH lesions from 6/12 (50%) subjects demonstrated evidence of microsatellite alterations in patterns consistent with their being monoclonal or containing

Table I. Microsatellite Loci

Name	Repeat	Chromosomal location
MYCL1	tetra	1p32
D1S549	tetra	1q32-42
D2S123	di	2p15
D3S1298	di	3p24
D3S1255	di	3p24.2-25
D5S346	di	5q21-22
D7S486	di	7q31
D7S480	di	7q31.1
THO1	tetra	11p15.5
D11S35	di	11q22
TP53	di	17p13.1
D17S579	di	17q21
D18S34	di	18q12.2-12.3
AR	tri	Xq11.2-12
HPRT	tetra	Xq26.1

Table II. Clonal Allelic Alterations Detected in AH Lesions

	AH lesions		
Subject	No. examined	No. monoclonal	Altered loci
10	1	—	
11	1	1	THO1 (v); MYCL1 (v)
12	2	—	
13	1	1	THO1 (v); D1S549 (1);
14	2	1	D3S1298 (v)
16	1	—	
17	1	1	AR (1); D1S549 (1); TP53 (v)
18	2	1	TP53 (1)
19	1	—	
20	1	—	
21	1	—	
23	1	1	D1S549 (v); D17S579 (v)
Total 12	15	6	

v, variation; *l*, loss.

a substantial monoclonal component. Both repeat length variation and allelic imbalance, most likely LOH, were detected. Table II summarizes the allelic alterations detected in all AH lesions examined. Because a microsatellite alteration that occurs in a cell that does not undergo clonal expansion should not be detected among the large excess of normal DNA from surrounding cells, alterations at even a single locus are evidence of monoclonality. Two AH lesions demonstrated alterations at a single locus. One (subject 14) showed evidence of length variation at D3S1298, and another (subject 18) showed evidence of allele loss at TP53 (data not shown). Three AH lesions demonstrated microsatellite alterations at two loci. Two of these lesions (from subjects 11 and 23) demonstrated microsatellite length variations exclusively, and the third (from subject 13) demonstrated both microsatellite length variation and LOH. Subject 11 appeared to have abnormalities in additional microsatellites, but the limited amounts of DNA available prevented unequivocal confirmation of those abnormalities. One AH lesion (subject 17) demonstrated alterations at three microsatellite loci, with evidence of both length alteration and allele loss. Fig. 1 illustrates the alterations seen in the four subjects with at least two microsatellite abnormalities. Detection of multiple altered loci in a single lesion diminishes the possibility that mosaicism or spontaneous mutation is responsible for the observed changes.

Although the sample size was small, and some potential subjects had to be eliminated from study for technical reasons (see Methods), we sought to identify pathologic or clinical features associated with evidence of monoclonality. Blinded review of the pathologic material revealed no correlation between histologic parameters and presence of monoclonality. Using available medical records, we determined each subject's age, race, personal or family history of breast pathology, and whether breast cancer had been diagnosed since the biopsy. Table III summarizes the clinical characteristics of the subjects studied. An association between monoclonality of the AH lesions and younger age was found: the mean age of the subjects who had AH lesions shown to be monoclonal was 44 yr (SD = 10.9), whereas that of subjects who had AH lesions without demonstrable monoclonality was 59 yr (SD = 10.6) ($P < 0.05$). There is also a suggestion that monoclonal AH lesions may be

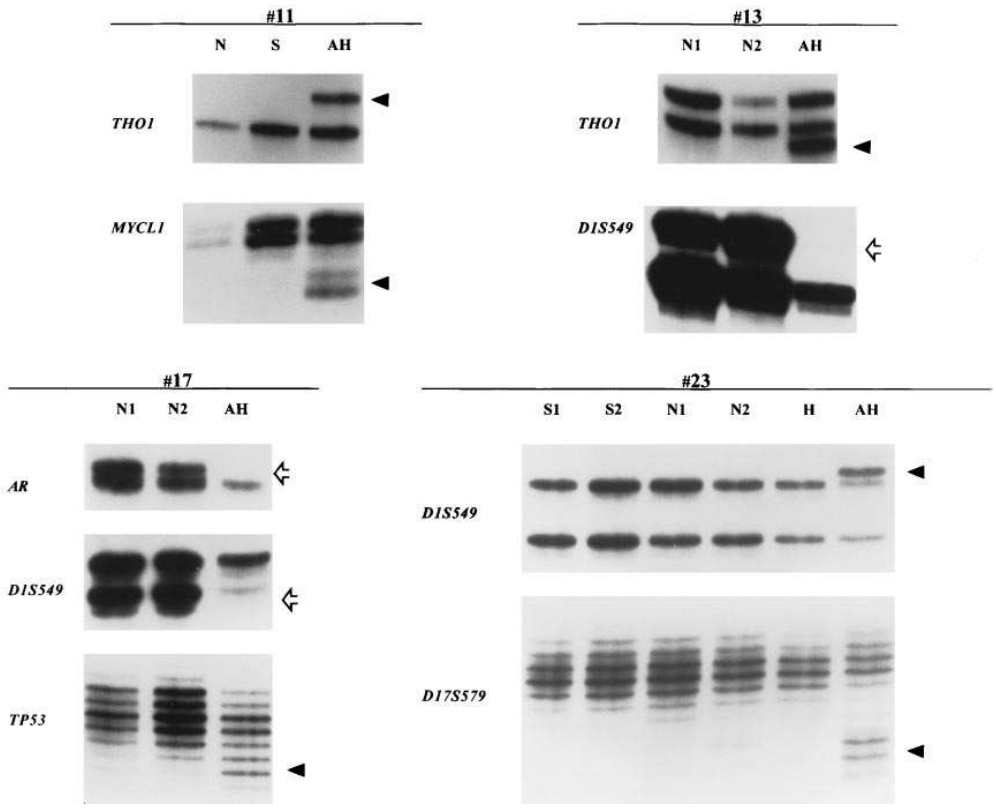

Figure 1. Microsatellite alterations seen in subjects 11, 13, 17, and 23, each shown in a separate panel. *S*, stroma; *N*, normal ductal epithelium; and *H*, hyperplastic lesion (without atypia). When more than one independent control sample was studied, each sample was numbered (i.e., N1, N2, or S1, S2). Names of the altered microsatellite loci are indicated at the left of each panel. Closed arrowheads indicate novel, AH-specific microsatellite alleles, open arrows indicate AH-specific loss of an allele.

Table III. Clinical Characteristics

Subject	Yr AH dx	Age (yr)	Breast-related history	Follow-up
Monoclonal AH				
11	1989	45		Lost
13	1987	37	Fibrocystic disease 1977; mother: breast cancer	Lost
14	1988	35	Aunt: breast cancer	No breast ds 1991
17	1991	51	Fibrocystic disease 1987	No breast ds 1994
18	1991	31		Fibroadenoma 1992; no breast ds 1995
23	1994	63		No breast ds 1995
		Avg: 44*		
AH lacking detectable monoclonality				
10	1994	58		No breast ds 1995
12	1989	63		No breast ds 1995
16	1987	59		Papillary breast epithelium 1992; no breast ds 1995
19	1991	41	Mother: breast cancer	Lost
20	1992	74		Lost
21	1991	59		Lost
		Avg: 59‡		

*SD = 10.9; ‡SD = 10.6; $P < 0.05$, Student's *t* test; *ds*, disease.

found more commonly in women with personal or family histories of breast disease. To date, no subjects studied here have developed a breast malignancy, although several patients have been lost to follow-up, and over half of the biopsies analyzed were performed within the last 5 yr. Black and white women were evenly distributed between the two groups.

Discussion

Alterations of short, highly polymorphic nucleotide repeat sequences, known as microsatellites, have been found to characterize many types of human tumors, including those of the breast (10–16). Recently, microsatellite alterations have been proposed as markers of clonality; the same microsatellite alterations found in a malignancy were also identified in apparently normal adjacent tissues, indicating the presence of unrecognized malignant cells (6). Monoclonality is a characteristic of neoplastic tissue, indicating that a cell has acquired abnormalities permitting escape from normal growth controls. In this study, we wished to determine whether monoclonal microsatellite alterations could be identified in proliferative breast lesions conventionally thought to be benign but associated with an increased risk of development of breast cancer. Traditional methods of demonstrating clonality based on X-inactivation (19, 20) can be difficult when the number of cells available is small (as from a single duct), particularly if the tissue has been fixed and paraffin-embedded. In addition, the normal clonal patch size arising from a single stem cell in mammary epithelium is not known. Therefore, we used a PCR-based technique to examine whether alterations in the normal pattern of up to 15 microsatellite sequences could be found in DNA extracted from microdissected AH lesions. This approach is feasible because the incidence of isolated background microsatellite alterations is believed to be low (5, 6), and in general, detection of novel expansions or deletions appears restricted to monoclonal tissue (6).

We have detected microsatellite alterations, both length variation and allele loss, indicating that at least a subset of the histologically benign AH lesions in fact contains a monoclonal cell population. This represents a minimum estimate of the fraction of AH lesions that are monoclonal, and study of a larger, or different, set of microsatellite loci, or use of other techniques, might increase the frequency of detection of monoclonality. Identification of monoclonal AH lesions implies that the process of neoplastic transformation and tumorigenesis may have already begun while breast tissue still appears histologically benign. Our data lend support to one proposed model of breast tumorigenesis in which carcinomas are postulated to arise from proliferative lesions as genetic abnormalities accumulate. Confirmation of this model would require demonstration that the same genetic abnormalities are present in both an AH lesion and the associated cancer. Consequently, it is premature to reclassify AH lesions as a type of carcinoma in situ, whose classification and subtyping itself is currently being debated (21). However, our findings do not require AH lesions to be obligate precursors of malignancy. It is quite possible that multiple monoclonal AH lesions could arise in a single subject, few, if any, of which ever accumulate the additional genetic abnormalities required to progress to a malignancy. This scenario suggests that a field defect might exist in certain breasts, a hypothesis consistent with the bilateral risk of cancer in patients diagnosed with AH lesions (1, 3, 17, 22, 23). Alternative models of breast tumorigenesis exist, and some breast tumors may not evolve through an identifiable proliferative stage.

There are few investigations of AH lesions in the literature (24–30). It is noteworthy that most studies have examined AH lesions present in an already cancerous breast; such lesions may not be directly comparable with the AH lesions we have

studied, which occurred in breasts lacking coincident or previous malignancies. However, our findings are compatible with those reports, using other methodologies, which suggest some proliferative breast lesions may be genetically abnormal (24, 25, 27–29). Other reports, also using different methodologies, have failed to find firm evidence of abnormalities (26, 30). This may reflect the possibility that AH lesions are genetically heterogeneous, or, that different methodologies detect abnormalities that occur at different stages in the process of tumorigenesis.

The technique used in this study suggests that some type of genetic instability may be an early event in the evolution of some breast neoplasms. Classical microsatellite instability, associated with defects in mismatch repair genes, is characterized by laddering of dinucleotide repeats at numerous loci (7–9). In contrast, sporadic tumors, including breast malignancies, tend to be characterized by microsatellite alterations at far fewer loci, which are often tri- or tetra- rather than dinucleotide repeat sequences (6, 11, 16); this pattern may reflect a more subtle or distinct repair defect (11). Our findings of relatively few loci affected within a given lesion, coupled with 4 of the total 11 alterations suggesting LOH, are more consistent with a defect(s) other than a mismatch repair gene abnormality being responsible for the changes described here. Some of the affected microsatellites may identify loci that are pathogenetically relevant, while others are more likely markers of an underlying genetic defect(s). It is unknown if this defect(s) is the same as that found in the 10–20% of breast cancers demonstrating microsatellite length variations (10–16), although there are suggestions that microsatellite alterations in breast cancer may occur as an early event (10, 12). We have found a higher overall frequency of microsatellite alterations in AH lesions than has been reported previously in breast cancer, and there are several possible explanations for this finding, including (*a*) our study examined a larger number of microsatellite loci, including some which were chosen to identify potential LOH; (*b*) selection of microsatellites more likely to be altered in breast tissue (some microsatellites appear to be altered in a tissue-specific pattern [6, 16]); and (*c*) some AH lesions examined in this study, although appearing ductal, may actually have been of lobular histology (lobular carcinomas have been associated with a higher [40%] incidence of microsatellite instability) (14).

Approximately 15% of patients with AH lesions go on to develop breast malignancy. The risk appears to affect both breasts (1, 3, 17, 22, 23), and may be greatest within 10 yr of diagnosis (31) and in premenopausal women (2, 4). At present, no reliable pathologic characteristics can identify which subjects with AH lesions will, and which will not, develop a malignancy. The percentage of AH lesions we found to be monoclonal is higher than the percentage of women with AH lesions who go on to develop a malignancy. Therefore, detection of monoclonality alone is unlikely to predict perfectly which women are destined to develop breast cancer. However, monoclonality may identify a subset of women at special risk. The association of monoclonal lesions with younger age and, presumably, premenopausal status, both of which are already known to be risk factors for cancer development among subjects with AH lesions, suggests that determination of monoclonality is a feature of these lesions with potential prognostic value. Examination of AH lesions from a larger group of women, refinement of the selection of microsatellite markers, and longer follow-up may reveal important clinical correlations.

Acknowledgments

The authors wish to thank Drs. Andrew Arnold and Thomas Rothstein for critical reading of the manuscript, and Ms. Susie Smith for expert technical assistance.

This work was supported by grants T32 AI07309 (P.S. Larson), NCI RO1 CA50459 (D.V. Faller), K08 CA62179 (C.L. Rosenberg), and funds from the Boston City Hospital Fund for Excellence, and the Aid for Cancer Research.

References

1. Dupont, W.D., and D.L. Page. 1985. Risk factors for breast cancer in women with proliferative breast disease. *N. Engl. J. Med.* 312:146–151.

2. Carter, C.L., D.K. Corle, M.S. Micozzi, A. Schiatzkin, and P.R. Taylor. 1988. A prospective study of the development of breast cancer in 16,692 women with benign breast disease. *Am. J. Epidemiol.* 128:467–477.

3. Tavassoli, F.A., and H.J. Norris. 1990. A comparison of the results of long-term follow-up for atypical intraductal hyperplasia and intraductal hyperplasia of the breast. *Cancer.* 65:518–529.

4. London, S.J., J.L. Connolly, S.J. Schnitt, and G.A. Colditz. 1992. A prospective study of benign breast disease and the risk of breast cancer. *J. Am. Med. Assoc.* 267:941–944.

5. Hackman, P., G. Gabbani, A.-M. Osterholm, D. Hellgren, and B. Lambert. 1995. Spontaneous length variation in microsatellite DNA from human T-cell clones. *Genes Chromosomes Cancer.* 14:215–219.

6. Mao, L., D.J. Lee, M.S. Tockman, Y.S. Erozan, F. Askin, and D. Sidransky. 1994. Microsatellite alterations as clonal markers for the detection of human cancer. *Proc. Natl. Acad. Sci. USA.* 91:9871–9875.

7. Aaltonen, L.A., P. Peltomaki, F.S. Leach, P. Sistonen, L. Pylkkanen, J.-P. Mecklin, H. Jarvinen, S.M. Powell, J. Jen, S.R. Hamilton, et al. 1993. Clues to the pathogenesis of familial colorectal cancer. *Science (Wash. DC).* 260:812–816.

8. Ionov, Y., M.A. Peinado, S. Malkhosyan, D. Shibata, and M. Perucho. 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature (Lond.).* 363:558–561.

9. Thibodeau, S.N., G. Bren, and D. Schaid. 1993. Microsatellite instability in cancer of the proximal colon. *Science (Wash. DC).* 260:816–819.

10. Yee, C.J., N. Roodi, C.S. Verrier, and F.F. Parl. 1994. Microsatellite instability and loss of heterozygosity in breast cancer. *Cancer Res.* 54:1641–1644.

11. Wooster, R., A.-M. Cleton-Jansen, N. Collins, J. Mangion, R.S. Cornelis, C.S. Cooper, B.A. Gusterson, B.A.J. Ponder, A. von Deimling, O.D. Wiestler, et al. 1994. Instability of short tandem repeats (microsatellites) in human cancers. *Nat. Genet.* 6:152–156.

12. Patel, U., S. Grundfest-Broniatowski, M. Gupta, and S. Banerjee. 1994. Microsatellite instabilities at five chromosomes in primary breast tumors. *Oncogene.* 9:3695–3700.

13. Eyfjord, J.E., S. Thorlacius, M. Steinarsdottir, R. Valgardsdottir, H.M. Ogmundsdottir, and K. Anamthawat-Jonsson. 1995. p53 abnormalities and genomic instability in primary human breast carcinomas. *Cancer Res.* 55:646–651.

14. Aldaz, C.M., T. Chen, A. Sahin, J. Cunningham, and M. Bondy. 1995. Comparative allelotype of in situ and invasive human breast cancer: high frequency of microsatellite instability in lobular breast carcinoma. *Cancer Res.* 55: 3976–3981.

15. Contegiacomo, A., R. Palmirotta, L. De Marchis, C. Pizzi, P. Mastranzo, P. Delrio, G. Petrella, M. Figliolini, A.R. Bianco, L. Frati, et al. 1995. Microsatellite instability and pathological aspects of breast cancer. *Int. J. Cancer.* 64: 264–268.

16. Jonsson, M., O. Johannsson, and A. Borg. 1995. Infrequent occurrence of microsatellite instability in sporadic and familiar breast cancer. *Eur. J. Cancer.* 31A:2330–2334.

17. Page, D.L., W.D. Dupont, L.W. Rogers, and M.S. Rados. 1985. Atypical hyperplastic lesions of the female breast: a long-term follow-up study. *Cancer (Philadelphia).* 55:2698–2708.

18. Mashal, R.D., S.C. Lester, and J. Sklar. 1993. Clonal analysis by study of X chromosome inactivation in formalin-fixed paraffin-embedded tissue. *Cancer Res.* 53:4676–4679.

19. Vogelstein, B., E.R. Fearon, S.R. Hamilton, and A.P. Feinberg. 1985. Use of restriction fragment length polymorphism to determine the clonal origins of human tumors. *Science (Wash. DC).* 227:642–645.

20. Allen, R.C., H.Y. Zoghbi, A.B. Moseley, H.M. Rosenblatt, and J.W. Belmont. 1992. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen receptor gene correlates with X chromosome inactivation. *Am. J. Hum. Genet.* 51:1229–1239.

21. Silverstein, M.J., D.N. Poller, J.R. Waisman, W.J. Colburn, A. Barth, E.D. Gierson, B. Lewinsky, P. Gamagami, and D. Slamon. 1995. Prognostic classification of breast ductal carcinoma-in-situ. *Lancet.* 345:1154–1157.

22. Davis, H.H., M. Simons, and J.B. Davis. 1964. Cystic disease of the breast: relationship to carcinoma. *Cancer (Philadelphia).* 17:957–978.

23. Krieger, N., and R.A. Hiatt. 1992. Risk of breast cancer after benign breast diseases. Variation by histologic type, degree of atypia, age at biopsy, and length of follow-up. *Am. J. Epidemiol.* 135:619–631.

24. Noguchi, S., K. Motomura, H. Inaji, S. Imaoka, and H. Koyama. 1994. Clonal analysis of predominantly intraductal carcinoma and precancerous lesions of the breast by means of polymerase chain reaction. *Cancer Res.* 54:1849–1853.

25. O'Connell, P., V. Pekkel, S. Fuqua, C.K. Osborne, and D.C. Allred. 1994. Molecular genetic studies of early breast cancer evolution. *Breast Cancer Res. Treat.* 32:5–12.

26. Eriksson, E.T., H. Schimmelpenning, U. Aspenblad, A. Zetterberg, and G.U. Auer. 1994. Immunohistochemical expression of the mutant p53 protein and nuclear DNA content during the transition from benign to malignant breast disease. *Hum. Pathol.* 25:1228–1233.

27. Micale, M.A., D.W. Visscher, S.E. Gulino, and S.R. Wolman. 1994. Chromosomal aneuploidy in proliferative breast disease. *Hum. Pathol.* 25:29–35.

28. Dietrich, C.U., N. Pandis, M.R. Teixeira, G. Bardi, A.-M. Gerdes, J.A. Andersen, and S. Heim. 1995. Chromosome abnormalities in benign hyperproliferative disorders of epithelial and stromal breast tissue. *Int. J. Cancer.* 60:49–53.

29. Lakhani, S.R., N. Collins, M.R. Stratton, and J.P. Sloane. 1995. Atypical ductal hyperplasia of the breast: clonal proliferation with loss of heterozygosity on chromosomes 16q and 17p. *J. Clin. Pathol.* 48:611–615.

30. Lizard-Nacol, S., R. Lidereau, F. Collin, M. Arnal, L. Hahnel, P. Roignot, J. Cuisenier, and J. Guerrin. 1995. Benign breast disease: absence of genetic alterations at several loci implicated in breast cancer malignancy. *Cancer Res.* 55:4416–4419.

31. Dupont, W.D., and D.L. Page. 1989. Relative risk of breast cancer varies with time since diagnosis of atypical hyperplasia. *Hum. Pathol.* 20:723–725.